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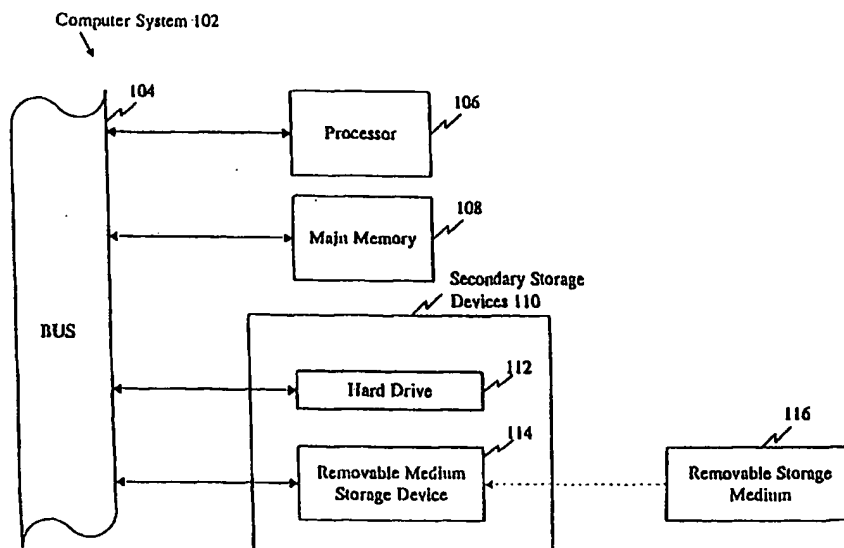


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(54) Title: *STREPTOCOCCUS PNEUMONIAE* POLYNUCLEOTIDES AND SEQUENCES



(57) Abstract

The present invention provides polynucleotide sequences of the genome of *Streptococcus pneumoniae*, polypeptide sequences encoded by the polynucleotide sequences, corresponding polynucleotides and polypeptides, vectors and hosts comprising the polynucleotides, and assays and other uses thereof. The present invention further provides polynucleotide and polypeptide sequence information stored on computer readable media, and computer-based systems and methods which facilitate its use.

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Streptococcus pneumoniae Polynucleotides and Sequences

FIELD OF THE INVENTION

5 The present invention relates to the field of molecular biology. In particular, it relates to, among other things, nucleotide sequences of *Streptococcus pneumoniae*, contigs, ORFs, fragments, probes, primers and related polynucleotides thereof, peptides and polypeptides encoded by the sequences, and uses of the polynucleotides and sequences thereof, such as in fermentation,
10 polypeptide production, assays and pharmaceutical development, among others.

BACKGROUND OF THE INVENTION

Streptococcus pneumoniae has been one of the most extensively studied
15 microorganisms since its first isolation in 1881. It was the object of many investigations that led to important scientific discoveries. In 1928, Griffith observed that when heat-killed encapsulated pneumococci and live strains constitutively lacking any capsule were concomitantly injected into mice, the nonencapsulated could be converted into encapsulated pneumococci with the same
20 capsular type as the heat-killed strain. Years later, the nature of this "transforming principle," or carrier of genetic information, was shown to be DNA. (Avery, O.T., et al., *J. Exp. Med.*, 79:137-157 (1944)).

 In spite of the vast number of publications on *S. pneumoniae* many questions about its virulence are still unanswered, and this pathogen remains a
25 major causative agent of serious human disease, especially community-acquired pneumonia. (Johnston, R.B., et al., *Rev. Infect. Dis.* 13(Suppl. 6):S509-517 (1991)). In addition, in developing countries, the pneumococcus is responsible for the death of a large number of children under the age of 5 years from pneumococcal pneumonia. The incidence of pneumococcal disease is highest in infants under 2
30 years of age and in people over 60 years of age. Pneumococci are the second most frequent cause (after *Haemophilus influenzae* type b) of bacterial meningitis and otitis media in children. With the recent introduction of conjugate vaccines for *H. influenzae* type b, pneumococcal meningitis is likely to become increasingly prominent. *S. pneumoniae* is the most important etiologic agent of community-

acquired pneumonia in adults and is the second most common cause of bacterial meningitis behind *Neisseria meningitidis*.

The antibiotic generally prescribed to treat *S. pneumoniae* is benzylpenicillin, although resistance to this and to other antibiotics is found occasionally. Pneumococcal resistance to penicillin results from mutations in its penicillin-binding proteins. In uncomplicated pneumococcal pneumonia caused by a sensitive strain, treatment with penicillin is usually successful unless started too late. Erythromycin or clindamycin can be used to treat pneumonia in patients hypersensitive to penicillin, but resistant strains to these drugs exist. Broad spectrum antibiotics (e.g., the tetracyclines) may also be effective, although tetracycline-resistant strains are not rare. In spite of the availability of antibiotics, the mortality of pneumococcal bacteremia in the last four decades has remained stable between 25 and 29%. (Gillespie, S.H., *et al.*, *J. Med. Microbiol.* 28:237-248 (1989).

S. pneumoniae is carried in the upper respiratory tract by many healthy individuals. It has been suggested that attachment of pneumococci is mediated by a disaccharide receptor on fibronectin, present on human pharyngeal epithelial cells. (Anderson, B.J., *et al.*, *J. Immunol.* 142:2464-2468 (1989). The mechanisms by which pneumococci translocate from the nasopharynx to the lung, thereby causing pneumonia, or migrate to the blood, giving rise to bacteremia or septicemia, are poorly understood. (Johnston, R.B., *et al.*, *Rev. Infect. Dis.* 13(Suppl. 6):S509-517 (1991).

Various proteins have been suggested to be involved in the pathogenicity of *S. pneumoniae*, however, only a few of them have actually been confirmed as virulence factors. Pneumococci produce an IgA1 protease that might interfere with host defense at mucosal surfaces. (Kornfield, S.J., *et al.*, *Rev. Inf. Dis.* 3:521-534 (1981). *S. pneumoniae* also produces neuraminidase, an enzyme that may facilitate attachment to epithelial cells by cleaving sialic acid from the host glycolipids and gangliosides. Partially purified neuraminidase was observed to induce meningitis-like symptoms in mice; however, the reliability of this finding has been questioned because the neuraminidase preparations used were probably contaminated with cell wall products. Other pneumococcal proteins besides neuraminidase are involved in the adhesion of pneumococci to epithelial and endothelial cells. These pneumococcal proteins have as yet not been identified. Recently, Cundell *et al.*, reported that peptide permeases can modulate

pneumococcal adherence to epithelial and endothelial cells. It was, however, unclear whether these permeases function directly as adhesions or whether they enhance adherence by modulating the expression of pneumococcal adhesions. (DeVelasco, E.A., *et al.*, *Micro. Rev.* 59:591-603 (1995). A better understanding of the virulence factors determining its pathogenicity will need to be developed to cope with the devastating effects of pneumococcal disease in humans.

Ironically, despite the prominent role of *S. pneumoniae* in the discovery of DNA, little is known about the molecular genetics of the organism. The *S. pneumoniae* genome consists of one circular, covalently closed, double-stranded DNA and a collection of so-called variable accessory elements, such as prophages, plasmids, transposons and the like. Most physical characteristics and almost all of the genes of *S. pneumoniae* are unknown. Among the few that have been identified, most have not been physically mapped or characterized in detail. Only a few genes of this organism have been sequenced. (See, for instance current versions of GENBANK and other nucleic acid databases, and references that relate to the genome of *S. pneumoniae* such as those set out elsewhere herein.)

It is clear that the etiology of diseases mediated or exacerbated by *S. pneumoniae*, infection involves the programmed expression of *S. pneumoniae* genes, and that characterizing the genes and their patterns of expression would add dramatically to our understanding of the organism and its host interactions. Knowledge of *S. pneumoniae* genes and genomic organization would improve our understanding of disease etiology and lead to improved and new ways of preventing, ameliorating, arresting and reversing diseases. Moreover, characterized genes and genomic fragments of *S. pneumoniae* would provide reagents for, among other things, detecting, characterizing and controlling *S. pneumoniae* infections. There is a need to characterize the genome of *S. pneumoniae* and for polynucleotides of this organism.

SUMMARY OF THE INVENTION

5 The present invention is based on the sequencing of fragments of the *Streptococcus pneumoniae* genome. The primary nucleotide sequences which were generated are provided in SEQ ID NOS:1-391.

The present invention provides the nucleotide sequence of several hundred contigs of the *Streptococcus pneumoniae* genome, which are listed in tables below and set out in the Sequence Listing submitted herewith, and representative
10 fragments thereof, in a form which can be readily used, analyzed, and interpreted by a skilled artisan. In one embodiment, the present invention is provided as contiguous strings of primary sequence information corresponding to the nucleotide sequences depicted in SEQ ID NOS:1-391.

The present invention further provides nucleotide sequences which are at
15 least 95% identical to the nucleotide sequences of SEQ ID NOS:1-391.

The nucleotide sequence of SEQ ID NOS:1-391, a representative fragment thereof, or a nucleotide sequence which is at least 95% identical to the nucleotide sequence of SEQ ID NOS:1-391 may be provided in a variety of mediums to facilitate its use. In one application of this embodiment, the sequences of the
20 present invention are recorded on computer readable media. Such media includes, but is not limited to: magnetic storage media, such as floppy discs, hard disc storage medium, and magnetic tape; optical storage media such as CD-ROM; electrical storage media such as RAM and ROM; and hybrids of these categories such as magnetic/optical storage media.

25 The present invention further provides systems, particularly computer-based systems which contain the sequence information herein described stored in a data storage means. Such systems are designed to identify commercially important fragments of the *Streptococcus pneumoniae* genome.

Another embodiment of the present invention is directed to fragments of the
30 *Streptococcus pneumoniae* genome having particular structural or functional attributes. Such fragments of the *Streptococcus pneumoniae* genome of the present invention include, but are not limited to, fragments which encode peptides, hereinafter referred to as open reading frames or ORFs, fragments which modulate the expression of an operably linked ORF, hereinafter referred to as expression
35 modulating fragments or EMFs, and fragments which can be used to diagnose the

presence of *Streptococcus pneumoniae* in a sample, hereinafter referred to as diagnostic fragments or DFs.

Each of the ORFs in fragments of the *Streptococcus pneumoniae* genome disclosed in Tables 1-3, and the EMFs found 5' to the ORFs, can be used in numerous ways as polynucleotide reagents. For instance, the sequences can be used as diagnostic probes or amplification primers for detecting or determining the presence of a specific microbe in a sample, to selectively control gene expression in a host and in the production of polypeptides, such as polypeptides encoded by ORFs of the present invention, particular those polypeptides that have a pharmacological activity.

The present invention further includes recombinant constructs comprising one or more fragments of the *Streptococcus pneumoniae* genome of the present invention. The recombinant constructs of the present invention comprise vectors, such as a plasmid or viral vector, into which a fragment of the *Streptococcus pneumoniae* has been inserted.

The present invention further provides host cells containing any of the isolated fragments of the *Streptococcus pneumoniae* genome of the present invention. The host cells can be a higher eukaryotic host cell, such as a mammalian cell, a lower eukaryotic cell, such as a yeast cell, or a procaryotic cell such as a bacterial cell.

The present invention is further directed to isolated polypeptides and proteins encoded by ORFs of the present invention. A variety of methods, well known to those of skill in the art, routinely may be utilized to obtain any of the polypeptides and proteins of the present invention. For instance, polypeptides and proteins of the present invention having relatively short, simple amino acid sequences readily can be synthesized using commercially available automated peptide synthesizers. Polypeptides and proteins of the present invention also may be purified from bacterial cells which naturally produce the protein. Yet another alternative is to purify polypeptide and proteins of the present invention from cells which have been altered to express them.

The invention further provides methods of obtaining homologs of the fragments of the *Streptococcus pneumoniae* genome of the present invention and homologs of the proteins encoded by the ORFs of the present invention. Specifically, by using the nucleotide and amino acid sequences disclosed herein as

a probe or as primers, and techniques such as PCR cloning and colony/plaque hybridization, one skilled in the art can obtain homologs.

The invention further provides antibodies which selectively bind polypeptides and proteins of the present invention. Such antibodies include both
5 monoclonal and polyclonal antibodies.

The invention further provides hybridomas which produce the above-described antibodies. A hybridoma is an immortalized cell line which is capable of secreting a specific monoclonal antibody.

The present invention further provides methods of identifying test samples
10 derived from cells which express one of the ORFs of the present invention, or a homolog thereof. Such methods comprise incubating a test sample with one or more of the antibodies of the present invention, or one or more of the DFs of the present invention, under conditions which allow a skilled artisan to determine if the sample contains the ORF or product produced therefrom.

15 In another embodiment of the present invention, kits are provided which contain the necessary reagents to carry out the above-described assays.

Specifically, the invention provides a compartmentalized kit to receive, in close confinement, one or more containers which comprises: (a) a first container comprising one of the antibodies, or one of the DFs of the present invention; and
20 (b) one or more other containers comprising one or more of the following: wash reagents, reagents capable of detecting presence of bound antibodies or hybridized DFs.

Using the isolated proteins of the present invention, the present invention further provides methods of obtaining and identifying agents capable of binding to
25 a polypeptide or protein encoded by one of the ORFs of the present invention. Specifically, such agents include, as further described below, antibodies, peptides, carbohydrates, pharmaceutical agents and the like. Such methods comprise steps of: (a) contacting an agent with an isolated protein encoded by one of the ORFs of the present invention; and (b) determining whether the agent binds to said protein.

30 The present genomic sequences of *Streptococcus pneumoniae* will be of great value to all laboratories working with this organism and for a variety of commercial purposes. Many fragments of the *Streptococcus pneumoniae* genome will be immediately identified by similarity searches against GenBank or protein databases and will be of immediate value to *Streptococcus pneumoniae* researchers

and for immediate commercial value for the production of proteins or to control gene expression.

The methodology and technology for elucidating extensive genomic sequences of bacterial and other genomes has and will greatly enhance the ability to
5 analyze and understand chromosomal organization. In particular, sequenced contigs and genomes will provide the models for developing tools for the analysis of chromosome structure and function, including the ability to identify genes within large segments of genomic DNA, the structure, position, and spacing of regulatory elements, the identification of genes with potential industrial applications, and the
10 ability to do comparative genomic and molecular phylogeny.

DESCRIPTION OF THE FIGURES

FIGURE 1 is a block diagram of a computer system (102) that can be
15 used to implement computer-based systems of present invention.

FIGURE 2 is a schematic diagram depicting the data flow and computer programs used to collect, assemble, edit and annotate the contigs of the *Streptococcus pneumoniae* genome of the present invention. Both Macintosh and
20 Unix platforms are used to handle the AB 373 and 377 sequence data files, largely as described in Kerlavage *et al.*, *Proceedings of the Twenty-Sixth Annual Hawaii International Conference on System Sciences*, 585, IEEE Computer Society Press, Washington D.C. (1993). Factura (AB) is a Macintosh program designed for automatic vector sequence removal and end-trimming of sequence files. The
25 program Loadis runs on a Macintosh platform and parses the feature data extracted from the sequence files by Factura to the Unix based *Streptococcus pneumoniae* relational database. Assembly of contigs (and whole genome sequences) is accomplished by retrieving a specific set of sequence files and their associated features using Extrseq, a Unix utility for retrieving sequences from an SQL
30 database. The resulting sequence file is processed by seq_filter to trim portions of the sequences with more than 2% ambiguous nucleotides. The sequence files were assembled using TIGR Assembler, an assembly engine designed at The Institute for Genomic Research (TIGR) for rapid and accurate assembly of thousands of sequence fragments. The collection of contigs generated by the assembly step is
35 loaded into the database with the lassie program. Identification of open reading

frames (ORFs) is accomplished by processing contigs with zorf or GenMark. The ORFs are searched against *S. pneumoniae* sequences from GenBank and against all protein sequences using the BLASTN and BLASTP programs, described in Altschul *et al.*, *J. Mol. Biol.* 215: 403-410 (1990)). Results of the ORF
5 determination and similarity searching steps were loaded into the database. As described below, some results of the determination and the searches are set out in Tables 1-3.

DETAILED DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

10

The present invention is based on the sequencing of fragments of the *Streptococcus pneumoniae* genome and analysis of the sequences. The primary nucleotide sequences generated by sequencing the fragments are provided in SEQ ID NOS:1-391. (As used herein, the "primary sequence" refers to the nucleotide
15 sequence represented by the IUPAC nomenclature system.)

In addition to the aforementioned *Streptococcus pneumoniae* polynucleotide and polynucleotide sequences, the present invention provides the nucleotide sequences of SEQ ID NOS:1-391, or representative fragments thereof, in a form which can be readily used, analyzed, and interpreted by a skilled artisan.

20 As used herein, a "representative fragment of the nucleotide sequence depicted in SEQ ID NOS:1-391" refers to any portion of the SEQ ID NOS:1-391 which is not presently represented within a publicly available database. Preferred representative fragments of the present invention are *Streptococcus pneumoniae* open reading frames (ORFs), expression modulating fragment (EMFs) and
25 fragments which can be used to diagnose the presence of *Streptococcus pneumoniae* in sample (DFs): A non-limiting identification of preferred representative fragments is provided in Tables 1-3. As discussed in detail below, the information provided in SEQ ID NOS:1-391 and in Tables 1-3 together with routine cloning, synthesis, sequencing and assay methods will enable those skilled
30 in the art to clone and sequence all "representative fragments" of interest, including open reading frames encoding a large variety of *Streptococcus pneumoniae* proteins.

While the presently disclosed sequences of SEQ ID NOS:1-391 are highly accurate, sequencing techniques are not perfect and, in relatively rare instances,
35 further investigation of a fragment or sequence of the invention may reveal a

nucleotide sequence error present in a nucleotide sequence disclosed in SEQ ID NOS:1-391. However, once the present invention is made available (*i.e.*, once the information in SEQ ID NOS:1-391 and Tables 1-3 has been made available), resolving a rare sequencing error in SEQ ID NOS:1-391 will be well within the skill of the art. The present disclosure makes available sufficient sequence information to allow any of the described contigs or portions thereof to be obtained readily by straightforward application of routine techniques. Further sequencing of such polynucleotide may proceed in like manner using manual and automated sequencing methods which are employed ubiquitous in the art. Nucleotide sequence editing software is publicly available. For example, Applied Biosystem's (AB) AutoAssembler can be used as an aid during visual inspection of nucleotide sequences. By employing such routine techniques potential errors readily may be identified and the correct sequence then may be ascertained by targeting further sequencing effort, also of a routine nature, to the region containing the potential error.

Even if all of the very rare sequencing errors in SEQ ID NOS:1-391 were corrected, the resulting nucleotide sequences would still be at least 95% identical, nearly all would be at least 99% identical, and the great majority would be at least 99.9% identical to the nucleotide sequences of SEQ ID NOS:1-391.

As discussed elsewhere herein, polynucleotides of the present invention readily may be obtained by routine application of well known and standard procedures for cloning and sequencing DNA. Detailed methods for obtaining libraries and for sequencing are provided below, for instance. A wide variety of *Streptococcus pneumoniae* strains that can be used to prepare *S. pneumoniae* genomic DNA for cloning and for obtaining polynucleotides of the present invention are available to the public from recognized depository institutions, such as the American Type Culture Collection (ATCC). While the present invention is enabled by the sequences and other information herein disclosed, the *S. pneumoniae* strain that provided the DNA of the present Sequence Listing, Strain 7/87 14.8.91, has been deposited in the ATCC, as a convenience to those of skill in the art. As a further convenience, a library of *S. pneumoniae* genomic DNA, derived from the same strain, also has been deposited in the ATCC. The *S. pneumoniae* strain was deposited on October 10, 1996, and was given Deposit No. 55840, and the cDNA library was deposited on October 11, 1996 and was given Deposit No. 97755. The genomic fragments in the library are 15 to 20 kb

fragments generated by partial Sau3A1 digestion and they are inserted into the BamHI site in the well-known lambda-derived vector lambda DASH II (Stratagene, La Jolla, CA). The provision of the deposits is not a waiver of any rights of the inventors or their assignees in the present subject matter.

5 The nucleotide sequences of the genomes from different strains of *Streptococcus pneumoniae* differ somewhat. However, the nucleotide sequences of the genomes of all *Streptococcus pneumoniae* strains will be at least 95% identical, in corresponding part, to the nucleotide sequences provided in SEQ ID NOS:1-391. Nearly all will be at least 99% identical and the great majority will be
10 99.9% identical.

Thus, the present invention further provides nucleotide sequences which are at least 95%, preferably 99% and most preferably 99.9% identical to the nucleotide sequences of SEQ ID NOS:1-391, in a form which can be readily used, analyzed and interpreted by the skilled artisan.

15 Methods for determining whether a nucleotide sequence is at least 95%, at least 99% or at least 99.9% identical to the nucleotide sequences of SEQ ID NOS:1-391 are routine and readily available to the skilled artisan. For example, the well known fasta algorithm described in Pearson and Lipman, *Proc. Natl. Acad. Sci. USA* 85: 2444 (1988) can be used to generate the percent identity of nucleotide
20 sequences. The BLASTN program also can be used to generate an identity score of polynucleotides compared to one another.

COMPUTER RELATED EMBODIMENTS

25 The nucleotide sequences provided in SEQ ID NOS:1-391, a representative fragment thereof, or a nucleotide sequence at least 95%, preferably at least 99% and most preferably at least 99.9% identical to a polynucleotide sequence of SEQ ID NOS:1-391 may be "provided" in a variety of mediums to facilitate use thereof. As used herein, provided refers to a manufacture, other than an isolated nucleic acid molecule, which contains a nucleotide sequence of the present invention; *i.e.*,
30 a nucleotide sequence provided in SEQ ID NOS:1-391, a representative fragment thereof, or a nucleotide sequence at least 95%, preferably at least 99% and most preferably at least 99.9% identical to a polynucleotide of SEQ ID NOS:1-391. Such a manufacture provides a large portion of the *Streptococcus pneumoniae* genome and parts thereof (*e.g.*, a *Streptococcus pneumoniae* open reading frame
35 (ORF)) in a form which allows a skilled artisan to examine the manufacture using

means not directly applicable to examining the *Streptococcus pneumoniae* genome or a subset thereof as it exists in nature or in purified form.

In one application of this embodiment, a nucleotide sequence of the present invention can be recorded on computer readable media. As used herein, "computer
5 readable media" refers to any medium which can be read and accessed directly by a computer. Such media include, but are not limited to: magnetic storage media, such as floppy discs, hard disc storage medium, and magnetic tape; optical storage media such as CD-ROM; electrical storage media such as RAM and ROM; and hybrids of these categories, such as magnetic/optical storage media. A skilled
10 artisan can readily appreciate how any of the presently known computer readable mediums can be used to create a manufacture comprising computer readable medium having recorded thereon a nucleotide sequence of the present invention. Likewise, it will be clear to those of skill how additional computer readable media that may be developed also can be used to create analogous manufactures having
15 recorded thereon a nucleotide sequence of the present invention.

As used herein, "recorded" refers to a process for storing information on computer readable medium. A skilled artisan can readily adopt any of the presently know methods for recording information on computer readable medium to generate manufactures comprising the nucleotide sequence information of the present
20 invention. A variety of data storage structures are available to a skilled artisan for creating a computer readable medium having recorded thereon a nucleotide sequence of the present invention. The choice of the data storage structure will generally be based on the means chosen to access the stored information. In addition, a variety of data processor programs and formats can be used to store the
25 nucleotide sequence information of the present invention on computer readable medium. The sequence information can be represented in a word processing text file, formatted in commercially-available software such as WordPerfect and MicroSoft Word, or represented in the form of an ASCII file, stored in a database application, such as DB2, Sybase, Oracle, or the like. A skilled artisan can readily
30 adapt any number of data-processor structuring formats (*e.g.*, text file or database) in order to obtain computer readable medium having recorded thereon the nucleotide sequence information of the present invention.

Computer software is publicly available which allows a skilled artisan to access sequence information provided in a computer readable medium. Thus, by
35 providing in computer readable form the nucleotide sequences of SEQ ID NOS:1-

391, a representative fragment thereof, or a nucleotide sequence at least 95%, preferably at least 99% and most preferably at least 99.9% identical to a sequence of SEQ ID NOS:1-391 the present invention enables the skilled artisan routinely to access the provided sequence information for a wide variety of purposes.

5 The examples which follow demonstrate how software which implements the BLAST (Altschul *et al.*, *J. Mol. Biol.* 215:403-410 (1990)) and BLAZE (Brutlag *et al.*, *Comp. Chem.* 17:203-207 (1993)) search algorithms on a Sybase system was used to identify open reading frames (ORFs) within the *Streptococcus pneumoniae* genome which contain homology to ORFs or proteins from both
10 *Streptococcus pneumoniae* and from other organisms. Among the ORFs discussed herein are protein encoding fragments of the *Streptococcus pneumoniae* genome useful in producing commercially important proteins, such as enzymes used in fermentation reactions and in the production of commercially useful metabolites.

15 The present invention further provides systems, particularly computer-based systems, which contain the sequence information described herein. Such systems are designed to identify, among other things, commercially important fragments of the *Streptococcus pneumoniae* genome.

20 As used herein, "a computer-based system" refers to the hardware means, software means, and data storage means used to analyze the nucleotide sequence information of the present invention. The minimum hardware means of the computer-based systems of the present invention comprises a central processing unit (CPU), input means, output means, and data storage means. A skilled artisan can readily appreciate that any one of the currently available computer-based systems are suitable for use in the present invention.

25 As stated above, the computer-based systems of the present invention comprise a data storage means having stored therein a nucleotide sequence of the present invention and the necessary hardware means and software means for supporting and implementing a search means.

30 As used herein, "data storage means" refers to memory which can store nucleotide sequence information of the present invention, or a memory access means which can access manufactures having recorded thereon the nucleotide sequence information of the present invention.

35 As used herein, "search means" refers to one or more programs which are implemented on the computer-based system to compare a target sequence or target structural motif with the sequence information stored within the data storage

means. Search means are used to identify fragments or regions of the present genomic sequences which match a particular target sequence or target motif. A variety of known algorithms are disclosed publicly and a variety of commercially available software for conducting search means are and can be used in the computer-based systems of the present invention. Examples of such software includes, but is not limited to, MacPattern (EMBL), BLASTN and BLASTX (NCBIA). A skilled artisan can readily recognize that any one of the available algorithms or implementing software packages for conducting homology searches can be adapted for use in the present computer-based systems.

As used herein, a "target sequence" can be any DNA or amino acid sequence of six or more nucleotides or two or more amino acids. A skilled artisan can readily recognize that the longer a target sequence is, the less likely a target sequence will be present as a random occurrence in the database. The most preferred sequence length of a target sequence is from about 10 to 100 amino acids or from about 30 to 300 nucleotide residues. However, it is well recognized that searches for commercially important fragments, such as sequence fragments involved in gene expression and protein processing, may be of shorter length.

As used herein, "a target structural motif," or "target motif," refers to any rationally selected sequence or combination of sequences in which the sequence(s) are chosen based on a three-dimensional configuration which is formed upon the folding of the target motif. There are a variety of target motifs known in the art. Protein target motifs include, but are not limited to, enzymic active sites and signal sequences. Nucleic acid target motifs include, but are not limited to, promoter sequences, hairpin structures and inducible expression elements (protein binding sequences).

A variety of structural formats for the input and output means can be used to input and output the information in the computer-based systems of the present invention. A preferred format for an output means ranks fragments of the *Streptococcus pneumoniae* genomic sequences possessing varying degrees of homology to the target sequence or target motif. Such presentation provides a skilled artisan with a ranking of sequences which contain various amounts of the target sequence or target motif and identifies the degree of homology contained in the identified fragment.

A variety of comparing means can be used to compare a target sequence or target motif with the data storage means to identify sequence fragments of the

Streptococcus pneumoniae genome. In the present examples, implementing software which implement the BLAST and BLAZE algorithms, described in Altschul *et al.*, *J. Mol. Biol.* 215: 403-410 (1990), is used to identify open reading frames within the *Streptococcus pneumoniae* genome. A skilled artisan can readily
5 recognize that any one of the publicly available homology search programs can be used as the search means for the computer-based systems of the present invention. Of course, suitable proprietary systems that may be known to those of skill also may be employed in this regard.

Figure 1 provides a block diagram of a computer system illustrative of
10 embodiments of this aspect of present invention. The computer system 102 includes a processor 106 connected to a bus 104. Also connected to the bus 104 are a main memory 108 (preferably implemented as random access memory, RAM) and a variety of secondary storage devices 110, such as a hard drive 112 and a removable medium storage device 114. The removable medium storage device 114
15 may represent, for example, a floppy disk drive, a CD-ROM drive, a magnetic tape drive, *etc.* A removable storage medium 116 (such as a floppy disk, a compact disk, a magnetic tape, *etc.*) containing control logic and/or data recorded therein may be inserted into the removable medium storage device 114. The computer system 102 includes appropriate software for reading the control logic and/or the
20 data from the removable medium storage device 114, once it is inserted into the removable medium storage device 114.

A nucleotide sequence of the present invention may be stored in a well known manner in the main memory 108, any of the secondary storage devices 110, and/or a removable storage medium 116. During execution, software for accessing
25 and processing the genomic sequence (such as search tools, comparing tools, *etc.*) reside in main memory 108, in accordance with the requirements and operating parameters of the operating system, the hardware system and the software program or programs.

BIOCHEMICAL EMBODIMENTS

Other embodiments of the present invention are directed to isolated fragments of the *Streptococcus pneumoniae* genome. The fragments of the
5 *Streptococcus pneumoniae* genome of the present invention include, but are not limited to fragments which encode peptides and polypeptides, hereinafter open reading frames (ORFs), fragments which modulate the expression of an operably linked ORF, hereinafter expression modulating fragments (EMFs) and fragments which can be used to diagnose the presence of *Streptococcus pneumoniae* in a
10 sample, hereinafter diagnostic fragments (DFs).

As used herein, an "isolated nucleic acid molecule" or an "isolated fragment of the *Streptococcus pneumoniae* genome" refers to a nucleic acid molecule possessing a specific nucleotide sequence which has been subjected to purification means to reduce, from the composition, the number of compounds which are
15 normally associated with the composition. Particularly, the term refers to the nucleic acid molecules having the sequences set out in SEQ ID NOS:1-391, to representative fragments thereof as described above, to polynucleotides at least 95%, preferably at least 99% and especially preferably at least 99.9% identical in sequence thereto, also as set out above.

20 A variety of purification means can be used to generate the isolated fragments of the present invention. These include, but are not limited to methods which separate constituents of a solution based on charge, solubility, or size.

In one embodiment, *Streptococcus pneumoniae* DNA can be enzymatically sheared to produce fragments of 15-20 kb in length. These fragments can then be
25 used to generate a *Streptococcus pneumoniae* library by inserting them into lambda clones as described in the Examples below. Primers flanking, for example, an ORF, such as those enumerated in Tables 1-3 can then be generated using nucleotide sequence information provided in SEQ ID NOS:1-391. Well known and routine techniques of PCR cloning then can be used to isolate the ORF from
30 the lambda DNA library or *Streptococcus pneumoniae* genomic DNA. Thus, given the availability of SEQ ID NOS:1-391, the information in Tables 1, 2 and 3, and the information that may be obtained readily by analysis of the sequences of SEQ ID NOS:1-391 using methods set out above, those of skill will be enabled by the present disclosure to isolate any ORF-containing or other nucleic acid fragment of
35 the present invention.

The isolated nucleic acid molecules of the present invention include, but are not limited to single stranded and double stranded DNA, and single stranded RNA.

As used herein, an "open reading frame," ORF, means a series of triplets coding for amino acids without any termination codons and is a sequence translatable into protein.

Tables 1, 2, and 3 list ORFs in the *Streptococcus pneumoniae* genomic contigs of the present invention that were identified as putative coding regions by the GeneMark software using organism-specific second-order Markov probability transition matrices. It will be appreciated that other criteria can be used, in accordance with well known analytical methods, such as those discussed herein, to generate more inclusive, more restrictive, or more selective lists.

Table 1 sets out ORFs in the *Streptococcus pneumoniae* contigs of the present invention that over a continuous region of at least 50 bases are 95% or more identical (by BLAST analysis) to a nucleotide sequence available through GenBank in October, 1997.

Table 2 sets out ORFs in the *Streptococcus pneumoniae* contigs of the present invention that are not in Table 1 and match, with a BLASTP probability score of 0.01 or less, a polypeptide sequence available through GenBank in October, 1997.

Table 3 sets out ORFs in the *Streptococcus pneumoniae* contigs of the present invention that do not match significantly, by BLASTP analysis, a polypeptide sequence available through GenBank in October, 1997.

In each table, the first and second columns identify the ORF by, respectively, contig number and ORF number within the contig; the third column indicates the first nucleotide of the ORF (actually the first nucleotide of the stop codon immediately preceeding the ORF), counting from the 5' end of the contig strand; and the fourth column, "stop (nt)" indicates the last nucleotide of the stop codon defining the 3' end of the ORF.

In Tables 1 and 2, column five, lists the Reference for the closest matching sequence available through GenBank. These reference numbers are the databases entry numbers commonly used by those of skill in the art, who will be familiar with their denominators. Descriptions of the nomenclature are available from the National Center for Biotechnology Information. Column six in Tables 1 and 2 provides the gene name of the matching sequence; column seven provides the BLAST identity score and column eight the BLAST similarity score from the

comparison of the ORF and the homologous gene; and column nine indicates the length in nucleotides of the highest scoring segment pair identified by the BLAST identity analysis.

Each ORF described in the tables is defined by "start (nt)" (5') and "stop (nt)" (3') nucleotide position numbers. These position numbers refer to the boundaries of each ORF and provide orientation with respect to whether the forward or reverse strand is the coding strand and which reading frame the coding sequence is contained. The "start" position is the first nucleotide of the triplet encoding a stop codon just 5' to the ORF and the "stop" position is the last nucleotide of the triplet encoding the next in-frame stop codon (i.e., the stop codon at the 3' end of the ORF). Those of ordinary skill in the art appreciate that preferred fragments within each ORF described in the table include fragments of each ORF which include the entire sequence from the delineated "start" and "stop" positions excepting the first and last three nucleotides since these encode stop codons. Thus, polynucleotides set out as ORFs in the tables but lacking the three (3) 5' nucleotides and the three (3) 3' nucleotides are encompassed by the present invention. Those of skill also appreciate that particularly preferred are fragments within each ORF that are polynucleotide fragments comprising polypeptide coding sequence. As defined herein, "coding sequence" includes the fragment within an ORF beginning at the first in-frame ATG (triplet encoding methionine) and ending with the last nucleotide prior to the triplet encoding the 3' stop codon. Preferred are fragments comprising the entire coding sequence and fragments comprising the entire coding sequence, excepting the coding sequence for the N-terminal methionine. Those of skill appreciate that the N-terminal methionine is often removed during post-translational processing and that polynucleotides lacking the ATG can be used to facilitate production of N-terminal fusion proteins which may be beneficial in the production or use of genetically engineered proteins. Of course, due to the degeneracy of the genetic code many polynucleotides can encode a given polypeptide. Thus, the invention further includes polynucleotides comprising a nucleotide sequence encoding a polypeptide sequence itself encoded by the coding sequence within an ORF described in Tables 1-3 herein. Further, polynucleotides at least 95%, preferably at least 99% and especially preferably at least 99.9% identical in sequence to the foregoing polynucleotides, are contemplated by the present invention.

Polypeptides encoded by polynucleotides described above and elsewhere herein are also provided by the present invention as are polypeptide comprising an amino acid sequence at least about 95%, preferably at least 97% and even more preferably 99% identical to the amino acid sequence of a polypeptide encoded by an ORF shown in Tables 1-3. These polypeptides may or may not comprise an N-terminal methionine.

The concepts of percent identity and percent similarity of two polypeptide sequences is well understood in the art. For example, two polypeptides 10 amino acids in length which differ at three amino acid positions (*e.g.*, at positions 1, 3 and 5) are said to have a percent identity of 70%. However, the same two polypeptides would be deemed to have a percent similarity of 80% if, for example at position 5, the amino acids moieties, although not identical, were "similar" (*i.e.*, possessed similar biochemical characteristics). Many programs for analysis of nucleotide or amino acid sequence similarity, such as fasta and BLAST specifically list percent identity of a matching region as an output parameter. Thus, for instance, Tables 1 and 2 herein enumerate the percent identity of the highest scoring segment pair in each ORF and its listed relative. Further details concerning the algorithms and criteria used for homology searches are provided below and are described in the pertinent literature highlighted by the citations provided below.

It will be appreciated that other criteria can be used to generate more inclusive and more exclusive listings of the types set out in the tables. As those of skill will appreciate, narrow and broad searches both are useful. Thus, a skilled artisan can readily identify ORFs in contigs of the *Streptococcus pneumoniae* genome other than those listed in Tables 1-3, such as ORFs which are overlapping or encoded by the opposite strand of an identified ORF in addition to those ascertainable using the computer-based systems of the present invention.

As used herein, an "expression modulating fragment," EMF, means a series of nucleotide molecules which modulates the expression of an operably linked ORF or EMF.

As used herein, a sequence is said to "modulate the expression of an operably linked sequence" when the expression of the sequence is altered by the presence of the EMF. EMFs include, but are not limited to, promoters, and promoter modulating sequences (inducible elements). One class of EMFs are
5 fragments which induce the expression of an operably linked ORF in response to a specific regulatory factor or physiological event.

EMF sequences can be identified within the contigs of the *Streptococcus pneumoniae* genome by their proximity to the ORFs provided in Tables 1-3. An intergenic segment, or a fragment of the intergenic segment, from about 10 to 200
10 nucleotides in length, taken from any one of the ORFs of Tables 1-3 will modulate the expression of an operably linked ORF in a fashion similar to that found with the naturally linked ORF sequence. As used herein, an "intergenic segment" refers to fragments of the *Streptococcus pneumoniae* genome which are between two ORF(s) herein described. EMFs also can be identified using known EMFs as a
15 target sequence or target motif in the computer-based systems of the present invention. Further, the two methods can be combined and used together.

The presence and activity of an EMF can be confirmed using an EMF trap vector. An EMF trap vector contains a cloning site linked to a marker sequence. A marker sequence encodes an identifiable phenotype, such as antibiotic resistance or
20 a complementing nutrition auxotrophic factor, which can be identified or assayed when the EMF trap vector is placed within an appropriate host under appropriate conditions. As described above, a EMF will modulate the expression of an operably linked marker sequence. A more detailed discussion of various marker sequences is provided below. A sequence which is suspected as being an EMF is
25 cloned in all three reading frames in one or more restriction sites upstream from the marker sequence in the EMF trap vector. The vector is then transformed into an appropriate host using known procedures and the phenotype of the transformed host is examined under appropriate conditions. As described above, an EMF will modulate the expression of an operably linked marker sequence.

30 As used herein, a "diagnostic fragment," DF, means a series of nucleotide molecules which selectively hybridize to *Streptococcus pneumoniae* sequences. DFs can be readily identified by identifying unique sequences within contigs of the *Streptococcus pneumoniae* genome, such as by using well-known computer analysis software, and by generating and testing probes or amplification primers

consisting of the DF sequence in an appropriate diagnostic format which determines amplification or hybridization selectivity.

The sequences falling within the scope of the present invention are not limited to the specific sequences herein described, but also include allelic and species variations thereof. Allelic and species variations can be routinely determined by comparing the sequences provided in SEQ ID NOS:1-391, a representative fragment thereof, or a nucleotide sequence at least 95%, preferably at least 99% and most at least preferably 99.9% identical to SEQ ID NOS:1-391, with a sequence from another isolate of the same species. Furthermore, to accommodate codon variability, the invention includes nucleic acid molecules coding for the same amino acid sequences as do the specific ORFs disclosed herein. In other words, in the coding region of an ORF, substitution of one codon for another which encodes the same amino acid is expressly contemplated. Any specific sequence disclosed herein can be readily screened for errors by resequencing a particular fragment, such as an ORF, in both directions (*i.e.*, sequence both strands). Alternatively, error screening can be performed by sequencing corresponding polynucleotides of *Streptococcus pneumoniae* origin isolated by using part or all of the fragments in question as a probe or primer.

Preferred DFs of the present invention comprise at least about 17, preferably at least about 20, and more preferably at least about 50 contiguous nucleotides within an ORF set out in Tables 1-3. Most highly preferred DFs specifically hybridize to a polynucleotide containing the sequence of the ORF from which they are derived. Specific hybridization occurs even under stringent conditions defined elsewhere herein.

Each of the ORFs of the *Streptococcus pneumoniae* genome disclosed in Tables 1, 2 and 3, and the EMFs found 5' to the ORFs, can be used as polynucleotide reagents in numerous ways. For example, the sequences can be used as diagnostic probes or diagnostic amplification primers to detect the presence of a specific microbe in a sample, particularly *Streptococcus pneumoniae*. Especially preferred in this regard are ORFs such as those of Table 3, which do not match previously characterized sequences from other organisms and thus are most likely to be highly selective for *Streptococcus pneumoniae*. Also particularly preferred are ORFs that can be used to distinguish between strains of *Streptococcus pneumoniae*, particularly those that distinguish medically important strain, such as drug-resistant strains.

In addition, the fragments of the present invention, as broadly described, can be used to control gene expression through triple helix formation or antisense DNA or RNA, both of which methods are based on the binding of a polynucleotide sequence to DNA or RNA. Triple helix-formation optimally results in a shut-off of RNA transcription from DNA, while antisense RNA hybridization blocks translation of an mRNA molecule into polypeptide. Information from the sequences of the present invention can be used to design antisense and triple helix-forming oligonucleotides. Polynucleotides suitable for use in these methods are usually 20 to 40 bases in length and are designed to be complementary to a region of the gene involved in transcription, for triple-helix formation, or to the mRNA itself, for antisense inhibition. Both techniques have been demonstrated to be effective in model systems, and the requisite techniques are well known and involve routine procedures. Triple helix techniques are discussed in, for example, Lee *et al.*, *Nucl. Acids Res.* 6:3073 (1979); Cooney *et al.*, *Science* 241:456 (1988); and Dervan *et al.*, *Science* 251:1360 (1991). Antisense techniques in general are discussed in, for instance, Okano, *J. Neurochem.* 56:560 (1991) and *Oligodeoxynucleotides as Antisense Inhibitors of Gene Expression*, CRC Press, Boca Raton, FL (1988)).

The present invention further provides recombinant constructs comprising one or more fragments of the *Streptococcus pneumoniae* genomic fragments and contigs of the present invention. Certain preferred recombinant constructs of the present invention comprise a vector, such as a plasmid or viral vector, into which a fragment of the *Streptococcus pneumoniae* genome has been inserted, in a forward or reverse orientation. In the case of a vector comprising one of the ORFs of the present invention, the vector may further comprise regulatory sequences, including for example, a promoter, operably linked to the ORF. For vectors comprising the EMFs of the present invention, the vector may further comprise a marker sequence or heterologous ORF operably linked to the EMF.

Large numbers of suitable vectors and promoters are known to those of skill in the art and are commercially available for generating the recombinant constructs of the present invention. The following vectors are provided by way of example. Useful bacterial vectors include phagescript, PsiX174, pBluescript SK, pBS KS, pNH8a, pNH16a, pNH18a, pNH46a (available from Stratagene); pTrc99A, pKK223-3, pKK233-3, pDR540, pRIT5 (available from Pharmacia). Useful eukaryotic vectors include pWLneo, pSV2cat, pOG44, pXT1, pSG

(available from Stratagene) pSVK3, pBPV, pMSG, pSVL (available from Pharmacia).

Promoter regions can be selected from any desired gene using CAT (chloramphenicol transferase) vectors or other vectors with selectable markers.

5 Two appropriate vectors are pKK232-8 and pCM7. Particular named bacterial promoters include lacI, lacZ, T3, T7, gpt, lambda PR, and trc. Eukaryotic promoters include CMV immediate early, HSV thymidine kinase, early and late SV40, LTRs from retrovirus, and mouse metallothionein- I. Selection of the appropriate vector and promoter is well within the level of ordinary skill in the art.

10 The present invention further provides host cells containing any one of the isolated fragments of the *Streptococcus pneumoniae* genomic fragments and contigs of the present invention, wherein the fragment has been introduced into the host cell using known methods. The host cell can be a higher eukaryotic host cell, such as a mammalian cell, a lower eukaryotic host cell, such as a yeast cell, or
15 a procaryotic cell, such as a bacterial cell.

A polynucleotide of the present invention, such as a recombinant construct comprising an ORF of the present invention, may be introduced into the host by a variety of well established techniques that are standard in the art, such as calcium phosphate transfection, DEAE, dextran mediated transfection and electroporation,
20 which are described in, for instance, Davis, L. *et al.*, BASIC METHODS IN MOLECULAR BIOLOGY (1986).

A host cell containing one of the fragments of the *Streptococcus pneumoniae* genomic fragments and contigs of the present invention, can be used in conventional manners to produce the gene product encoded by the isolated
25 fragment (in the case of an ORF) or can be used to produce a heterologous protein under the control of the EMF. The present invention further provides isolated polypeptides encoded by the nucleic acid fragments of the present invention or by degenerate variants of the nucleic acid fragments of the present invention. By "degenerate variant" is intended nucleotide fragments which differ
30 from a nucleic acid fragment of the present invention (*e.g.*, an ORF) by nucleotide sequence but, due to the degeneracy of the Genetic Code, encode an identical polypeptide sequence.

Preferred nucleic acid fragments of the present invention are the ORFs and subfragments thereof depicted in Tables 2 and 3 which encode proteins.

A variety of methodologies known in the art can be utilized to obtain any one of the isolated polypeptides or proteins of the present invention. At the simplest level, the amino acid sequence can be synthesized using commercially available peptide synthesizers. This is particularly useful in producing small peptides and fragments of larger polypeptides. Such short fragments as may be obtained most readily by synthesis are useful, for example, in generating antibodies against the native polypeptide, as discussed further below.

In an alternative method, the polypeptide or protein is purified from bacterial cells which naturally produce the polypeptide or protein. One skilled in the art can readily employ well-known methods for isolating polypeptides and proteins to isolate and purify polypeptides or proteins of the present invention produced naturally by a bacterial strain, or by other methods. Methods for isolation and purification that can be employed in this regard include, but are not limited to, immunochromatography, HPLC, size-exclusion chromatography, ion-exchange chromatography, and immuno-affinity chromatography.

The polypeptides and proteins of the present invention also can be purified from cells which have been altered to express the desired polypeptide or protein. As used herein, a cell is said to be altered to express a desired polypeptide or protein when the cell, through genetic manipulation, is made to produce a polypeptide or protein which it normally does not produce or which the cell normally produces at a lower level. Those skilled in the art can readily adapt procedures for introducing and expressing either recombinant or synthetic sequences into eukaryotic or prokaryotic cells in order to generate a cell which produces one of the polypeptides or proteins of the present invention.

Any host/vector system can be used to express one or more of the ORFs of the present invention. These include, but are not limited to, eukaryotic hosts such as HeLa cells, CV-1 cell, COS cells, and Sf9 cells, as well as prokaryotic host such as *E. coli* and *B. subtilis*. The most preferred cells are those which do not normally express the particular polypeptide or protein or which expresses the polypeptide or protein at low natural level.

"Recombinant," as used herein, means that a polypeptide or protein is derived from recombinant (*e.g.*, microbial or mammalian) expression systems. "Microbial" refers to recombinant polypeptides or proteins made in bacterial or fungal (*e.g.*, yeast) expression systems. As a product, "recombinant microbial" defines a polypeptide or protein essentially free of native endogenous substances and unaccompanied by associated native glycosylation. Polypeptides or proteins expressed in most bacterial cultures, *e.g.*, *E. coli*, will be free of glycosylation modifications; polypeptides or proteins expressed in yeast will have a glycosylation pattern different from that expressed in mammalian cells.

"Nucleotide sequence" refers to a heteropolymer of deoxyribonucleotides. Generally, DNA segments encoding the polypeptides and proteins provided by this invention are assembled from fragments of the *Streptococcus pneumoniae* genome and short oligonucleotide linkers, or from a series of oligonucleotides, to provide a synthetic gene which is capable of being expressed in a recombinant transcriptional unit comprising regulatory elements derived from a microbial or viral operon.

Recombinant expression vehicle or vector" refers to a plasmid or phage or virus or vector, for expressing a polypeptide from a DNA (RNA) sequence. The expression vehicle can comprise a transcriptional unit comprising an assembly of (1) a genetic regulatory elements necessary for gene expression in the host, including elements required to initiate and maintain transcription at a level sufficient for suitable expression of the desired polypeptide, including, for example, promoters and, where necessary, an enhancer and a polyadenylation signal; (2) a structural or coding sequence which is transcribed into mRNA and translated into protein, and (3) appropriate signals to initiate translation at the beginning of the desired coding region and terminate translation at its end. Structural units intended for use in yeast or eukaryotic expression systems preferably include a leader sequence enabling extracellular secretion of translated protein by a host cell. Alternatively, where recombinant protein is expressed without a leader or transport sequence, it may include an N-terminal methionine residue. This residue may or may not be subsequently cleaved from the expressed recombinant protein to provide a final product.

"Recombinant expression system" means host cells which have stably integrated a recombinant transcriptional unit into chromosomal DNA or carry the recombinant transcriptional unit extra chromosomally. The cells can be prokaryotic or eukaryotic. Recombinant expression systems as defined herein will express

heterologous polypeptides or proteins upon induction of the regulatory elements linked to the DNA segment or synthetic gene to be expressed.

Mature proteins can be expressed in mammalian cells, yeast, bacteria, or other cells under the control of appropriate promoters. Cell-free translation systems can also be employed to produce such proteins using RNAs derived from the DNA constructs of the present invention. Appropriate cloning and expression vectors for use with prokaryotic and eukaryotic hosts are described in Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, 2nd Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1989), the disclosure of which is hereby incorporated by reference in its entirety.

Generally, recombinant expression vectors will include origins of replication and selectable markers permitting transformation of the host cell, *e.g.*, the ampicillin resistance gene of *E. coli* and *S. cerevisiae* TRP1 gene, and a promoter derived from a highly expressed gene to direct transcription of a downstream structural sequence. Such promoters can be derived from operons encoding glycolytic enzymes such as 3-phosphoglycerate kinase (PGK), alpha-factor, acid phosphatase, or heat shock proteins, among others. The heterologous structural sequence is assembled in appropriate phase with translation initiation and termination sequences, and preferably, a leader sequence capable of directing secretion of translated protein into the periplasmic space or extracellular medium. Optionally, the heterologous sequence can encode a fusion protein including an N-terminal identification peptide imparting desired characteristics, *e.g.*, stabilization or simplified purification of expressed recombinant product.

Useful expression vectors for bacterial use are constructed by inserting a structural DNA sequence encoding a desired protein together with suitable translation initiation and termination signals in operable reading phase with a functional promoter. The vector will comprise one or more phenotypic selectable markers and an origin of replication to ensure maintenance of the vector and, when desirable, provide amplification within the host.

Suitable prokaryotic hosts for transformation include strains of *E. coli*, *B. subtilis*, *Salmonella typhimurium* and various species within the genera *Pseudomonas* and *Streptomyces*. Others may, also be employed as a matter of choice.

As a representative but non-limiting example, useful expression vectors for bacterial use can comprise a selectable marker and bacterial origin of replication

derived from commercially available plasmids comprising genetic elements of the well known cloning vector pBR322 (ATCC 37017). Such commercial vectors include, for example, pKK223-3 (available from Pharmacia Fine Chemicals, Uppsala, Sweden) and GEM 1 (available from Promega Biotec, Madison, WI, USA). These pBR322 "backbone" sections are combined with an appropriate promoter and the structural sequence to be expressed.

Following transformation of a suitable host strain and growth of the host strain to an appropriate cell density, the selected promoter, where it is inducible, is derepressed or induced by appropriate means (*e.g.*, temperature shift or chemical induction) and cells are cultured for an additional period to provide for expression of the induced gene product. Thereafter cells are typically harvested, generally by centrifugation, disrupted to release expressed protein, generally by physical or chemical means, and the resulting crude extract is retained for further purification.

Various mammalian cell culture systems can also be employed to express recombinant protein. Examples of mammalian expression systems include the COS-7 lines of monkey kidney fibroblasts, described in Gluzman, *Cell* 23:175 (1981), and other cell lines capable of expressing a compatible vector, for example, the C127, 3T3, CHO, HeLa and BHK cell lines.

Mammalian expression vectors will comprise an origin of replication, a suitable promoter and enhancer, and also any necessary ribosome binding sites, polyadenylation site, splice donor and acceptor sites, transcriptional termination sequences, and 5' flanking nontranscribed sequences. DNA sequences derived from the SV40 viral genome, for example, SV40 origin, early promoter, enhancer, splice, and polyadenylation sites may be used to provide the required nontranscribed genetic elements.

Recombinant polypeptides and proteins produced in bacterial culture is usually isolated by initial extraction from cell pellets, followed by one or more salting-out, aqueous ion exchange or size exclusion chromatography steps. Microbial cells employed in expression of proteins can be disrupted by any convenient method, including freeze-thaw cycling, sonication, mechanical disruption, or use of cell lysing agents. Protein refolding steps can be used, as necessary, in completing configuration of the mature protein. Finally, high performance liquid chromatography (HPLC) can be employed for final purification steps.

The present invention further includes isolated polypeptides, proteins and nucleic acid molecules which are substantially equivalent to those herein described. As used herein, substantially equivalent can refer both to nucleic acid and amino acid sequences, for example a mutant sequence, that varies from a reference sequence by one or more substitutions, deletions, or additions, the net effect of which does not result in an adverse functional dissimilarity between reference and subject sequences. For purposes of the present invention, sequences having equivalent biological activity, and equivalent expression characteristics are considered substantially equivalent. For purposes of determining equivalence, truncation of the mature sequence should be disregarded.

The invention further provides methods of obtaining homologs from other strains of *Streptococcus pneumoniae*, of the fragments of the *Streptococcus pneumoniae* genome of the present invention and homologs of the proteins encoded by the ORFs of the present invention. As used herein, a sequence or protein of *Streptococcus pneumoniae* is defined as a homolog of a fragment of the *Streptococcus pneumoniae* fragments or contigs or a protein encoded by one of the ORFs of the present invention, if it shares significant homology to one of the fragments of the *Streptococcus pneumoniae* genome of the present invention or a protein encoded by one of the ORFs of the present invention. Specifically, by using the sequence disclosed herein as a probe or as primers, and techniques such as PCR cloning and colony/plaque hybridization, one skilled in the art can obtain homologs.

As used herein, two nucleic acid molecules or proteins are said to "share significant homology" if the two contain regions which possess greater than 85% sequence (amino acid or nucleic acid) homology. Preferred homologs in this regard are those with more than 90% homology. Especially preferred are those with 93% or more homology. Among especially preferred homologs those with 95% or more homology are particularly preferred. Very particularly preferred among these are those with 97% and even more particularly preferred among those are homologs with 99% or more homology. The most preferred homologs among these are those with 99.9% homology or more. It will be understood that, among measures of homology, identity is particularly preferred in this regard.

Region specific primers or probes derived from the nucleotide sequence provided in SEQ ID NOS:1-391 or from a nucleotide sequence at least 95%, particularly at least 99%, especially at least 99.5% identical to a sequence of SEQ

ID NOS:1-391 can be used to prime DNA synthesis and PCR amplification, as well as to identify colonies containing cloned DNA encoding a homolog. Methods suitable to this aspect of the present invention are well known and have been described in great detail in many publications such as, for example, Innis *et al.*,
5 *PCR Protocols*, Academic Press, San Diego, CA (1990)).

When using primers derived from SEQ ID NOS:1-391 or from a nucleotide sequence having an aforementioned identity to a sequence of SEQ ID NOS:1-391, one skilled in the art will recognize that by employing high stringency conditions (*e.g.*, annealing at 50-60°C in 6X SSPE and 50% formamide, and washing at 50-
10 65°C in 0.5X SSPE) only sequences which are greater than 75% homologous to the primer will be amplified. By employing lower stringency conditions (*e.g.*, hybridizing at 35-37°C in 5X SSPE and 40-45% formamide, and washing at 42°C in 0.5X SSPE), sequences which are greater than 40-50% homologous to the primer will also be amplified.

15 When using DNA probes derived from SEQ ID NOS:1-391, or from a nucleotide sequence having an aforementioned identity to a sequence of SEQ ID NOS:1-391, for colony/plaque hybridization, one skilled in the art will recognize that by employing high stringency conditions (*e.g.*, hybridizing at 50- 65°C in 5X SSPE and 50% formamide, and washing at 50- 65°C in 0.5X SSPE), sequences
20 having regions which are greater than 90% homologous to the probe can be obtained, and that by employing lower stringency conditions (*e.g.*, hybridizing at 35-37°C in 5X SSPE and 40-45% formamide, and washing at 42°C in 0.5X SSPE), sequences having regions which are greater than 35-45% homologous to the probe will be obtained.

25 Any organism can be used as the source for homologs of the present invention so long as the organism naturally expresses such a protein or contains genes encoding the same. The most preferred organism for isolating homologs are bacteria which are closely related to *Streptococcus pneumoniae*.

30 ILLUSTRATIVE USES OF COMPOSITIONS OF THE INVENTION

Each ORF provided in Tables 1 and 2 is identified with a function by homology to a known gene or polypeptide. As a result, one skilled in the art can use the polypeptides of the present invention for commercial, therapeutic and
35 industrial purposes consistent with the type of putative identification of the

polypeptide. Such identifications permit one skilled in the art to use the *Streptococcus pneumoniae* ORFs in a manner similar to the known type of sequences for which the identification is made; for example, to ferment a particular sugar source or to produce a particular metabolite. A variety of reviews illustrative of this aspect of the invention are available, including the following reviews on the industrial use of enzymes, for example, BIOCHEMICAL ENGINEERING AND BIOTECHNOLOGY HANDBOOK, 2nd Ed., MacMillan Publications, Ltd. NY (1991) and BIOCATALYSTS IN ORGANIC SYNTHESSES, Tramper *et al.*, Eds., Elsevier Science Publishers, Amsterdam, The Netherlands (1985). A variety of exemplary uses that illustrate this and similar aspects of the present invention are discussed below.

1. Biosynthetic Enzymes

Open reading frames encoding proteins involved in mediating the catalytic reactions involved in intermediary and macromolecular metabolism, the biosynthesis of small molecules, cellular processes and other functions includes enzymes involved in the degradation of the intermediary products of metabolism, enzymes involved in central intermediary metabolism, enzymes involved in respiration, both aerobic and anaerobic, enzymes involved in fermentation, enzymes involved in ATP proton motor force conversion, enzymes involved in broad regulatory function, enzymes involved in amino acid synthesis, enzymes involved in nucleotide synthesis, enzymes involved in cofactor and vitamin synthesis, can be used for industrial biosynthesis.

The various metabolic pathways present in *Streptococcus pneumoniae* can be identified based on absolute nutritional requirements as well as by examining the various enzymes identified in Table 1-3 and SEQ ID NOS:1-391.

Of particular interest are polypeptides involved in the degradation of intermediary metabolites as well as non-macromolecular metabolism. Such enzymes include amylases, glucose oxidases, and catalase.

Proteolytic enzymes are another class of commercially important enzymes. Proteolytic enzymes find use in a number of industrial processes including the processing of flax and other vegetable fibers, in the extraction, clarification and depectinization of fruit juices, in the extraction of vegetables' oil and in the maceration of fruits and vegetables to give unicellular fruits. A detailed review of the proteolytic enzymes used in the food industry is provided in Rombouts *et al.*,

Symbiosis 21:79 (1986) and Voragen *et al.* in *Biocatalysts In Agricultural Biotechnology*, Whitaker *et al.*, Eds., *American Chemical Society Symposium Series* 389:93 (1989).

5 The metabolism of sugars is an important aspect of the primary metabolism of *Streptococcus pneumoniae*. Enzymes involved in the degradation of sugars, such as, particularly, glucose, galactose, fructose and xylose, can be used in industrial fermentation. Some of the important sugar transforming enzymes, from a commercial viewpoint, include sugar isomerases such as glucose isomerase. Other metabolic enzymes have found commercial use such as glucose oxidases
10 which produces ketogulonic acid (KGA). KGA is an intermediate in the commercial production of ascorbic acid using the Reichstein's procedure, as described in Krueger *et al.*, *Biotechnology* 6(A), Rhine *et al.*, Eds., Verlag Press, Weinheim, Germany (1984).

Glucose oxidase (GOD) is commercially available and has been used in
15 purified form as well as in an immobilized form for the deoxygenation of beer. See, for instance, Hartmeir *et al.*, *Biotechnology Letters* 1:21 (1979). The most important application of GOD is the industrial scale fermentation of gluconic acid. Market for gluconic acids which are used in the detergent, textile, leather, photographic, pharmaceutical, food, feed and concrete industry, as described, for
20 example, in Bigelis *et al.*, beginning on page 357 in *GENE MANIPULATIONS AND FUNGI*; Benett *et al.*, Eds., Academic Press, New York (1985). In addition to industrial applications, GOD has found applications in medicine for quantitative determination of glucose in body fluids recently in biotechnology for analyzing syrups from starch and cellulose hydrosylates. This application is described in
25 Owusu *et al.*, *Biochem. et Biophysica. Acta.* 872:83 (1986), for instance.

The main sweetener used in the world today is sugar which comes from sugar beets and sugar cane. In the field of industrial enzymes, the glucose isomerase process shows the largest expansion in the market today. Initially, soluble enzymes were used and later immobilized enzymes were developed
30 (Krueger *et al.*, *Biotechnology, The Textbook of Industrial Microbiology*, Sinauer Associated Incorporated, Sunderland, Massachusetts (1990)). Today, the use of glucose- produced high fructose syrups is by far the largest industrial business using immobilized enzymes. A review of the industrial use of these enzymes is provided by Jorgensen, *Starch* 40:307 (1988).

Proteinases, such as alkaline serine proteinases, are used as detergent additives and thus represent one of the largest volumes of microbial enzymes used in the industrial sector. Because of their industrial importance, there is a large body of published and unpublished information regarding the use of these enzymes in industrial processes. (See Faultman *et al.*, *Acid Proteases Structure Function and Biology*, Tang, J., ed., Plenum Press, New York (1977) and Godfrey *et al.*, *Industrial Enzymes*, MacMillan Publishers, Surrey, UK (1983) and Hepner *et al.*, *Report Industrial Enzymes by 1990*, Hel Hepner & Associates, London (1986)).

Another class of commercially usable proteins of the present invention are the microbial lipases, described by, for instance, Macrae *et al.*, *Philosophical Transactions of the Chiral Society of London* 310:227 (1985) and Poserke, *Journal of the American Oil Chemist Society* 61:1758 (1984). A major use of lipases is in the fat and oil industry for the production of neutral glycerides using lipase catalyzed inter-esterification of readily available triglycerides. Application of lipases include the use as a detergent additive to facilitate the removal of fats from fabrics in the course of the washing procedures.

The use of enzymes, and in particular microbial enzymes, as catalyst for key steps in the synthesis of complex organic molecules is gaining popularity at a great rate. One area of great interest is the preparation of chiral intermediates. Preparation of chiral intermediates is of interest to a wide range of synthetic chemists particularly those scientists involved with the preparation of new pharmaceuticals, agrochemicals, fragrances and flavors. (See Davies *et al.*, *Recent Advances in the Generation of Chiral Intermediates Using Enzymes*, CRC Press, Boca Raton, Florida (1990)). The following reactions catalyzed by enzymes are of interest to organic chemists: hydrolysis of carboxylic acid esters, phosphate esters, amides and nitriles, esterification reactions, trans-esterification reactions, synthesis of amides, reduction of alkanones and oxoalkanates, oxidation of alcohols to carbonyl compounds, oxidation of sulfides to sulfoxides, and carbon bond forming reactions such as the aldol reaction.

When considering the use of an enzyme encoded by one of the ORFs of the present invention for biotransformation and organic synthesis it is sometimes necessary to consider the respective advantages and disadvantages of using a microorganism as opposed to an isolated enzyme. Pros and cons of using a whole cell system on the one hand or an isolated partially purified enzyme on the other

hand, has been described in detail by Bud *et al.*, Chemistry in Britain (1987), p. 127.

Amino transferases, enzymes involved in the biosynthesis and metabolism of amino acids, are useful in the catalytic production of amino acids. The advantages of using microbial based enzyme systems is that the amino transferase enzymes catalyze the stereo- selective synthesis of only L-amino acids and generally possess uniformly high catalytic rates. A description of the use of amino transferases for amino acid production is provided by Roselle-David, *Methods of Enzymology* 136:479 (1987).

Another category of useful proteins encoded by the ORFs of the present invention include enzymes involved in nucleic acid synthesis, repair, and recombination.

2. Generation of Antibodies

As described here, the proteins of the present invention, as well as homologs thereof, can be used in a variety of procedures and methods known in the art which are currently applied to other proteins. The proteins of the present invention can further be used to generate an antibody which selectively binds the protein. Such antibodies can be either monoclonal or polyclonal antibodies, as well fragments of these antibodies, and humanized forms.

The invention further provides antibodies which selectively bind to one of the proteins of the present invention and hybridomas which produce these antibodies. A hybridoma is an immortalized cell line which is capable of secreting a specific monoclonal antibody.

In general, techniques for preparing polyclonal and monoclonal antibodies as well as hybridomas capable of producing the desired antibody are well known in the art (Campbell, A. M., *Monoclonal Antibody Technology: Laboratory Techniques In Biochemistry And Molecular Biology*, Elsevier Science Publishers, Amsterdam, The Netherlands (1984); St. Groth *et al.*, *J. Immunol. Methods* 35: 1-21 (1980), Kohler and Milstein, *Nature* 256:495-497 (1975)), the trioma technique, the human B-cell hybridoma technique (Kozbor *et al.*, *Immunology Today* 4:72 (1983), pgs. 77-96 of Cole *et al.*, in *Monoclonal Antibodies And Cancer Therapy*, Alan R. Liss, Inc. (1985)). Any animal (mouse, rabbit, etc.) which is known to produce antibodies can be immunized with the pseudogene polypeptide. Methods for immunization are well known in the art. Such methods

include subcutaneous or interperitoneal injection of the polypeptide. One skilled in the art will recognize that the amount of the protein encoded by the ORF of the present invention used for immunization will vary based on the animal which is immunized, the antigenicity of the peptide and the site of injection.

5 The protein which is used as an immunogen may be modified or administered in an adjuvant in order to increase the protein's antigenicity. Methods of increasing the antigenicity of a protein are well known in the art and include, but are not limited to coupling the antigen with a heterologous protein (such as globulin or galactosidase) or through the inclusion of an adjuvant during immunization.

10 For monoclonal antibodies, spleen cells from the immunized animals are removed, fused with myeloma cells, such as SP2/0-Ag14 myeloma cells, and allowed to become monoclonal antibody producing hybridoma cells.

Any one of a number of methods well known in the art can be used to identify the hybridoma cell which produces an antibody with the desired characteristics. These include screening the hybridomas with an ELISA assay, western blot analysis, or radioimmunoassay (Lutz *et al.*, *Exp. Cell Res.* 175:109-124 (1988)).

15 Hybridomas secreting the desired antibodies are cloned and the class and subclass is determined using procedures known in the art (Campbell, A. M., *Monoclonal Antibody Technology: Laboratory Techniques in Biochemistry and Molecular Biology*, Elsevier Science Publishers, Amsterdam, The Netherlands (1984)).

20 Techniques described for the production of single chain antibodies (U. S. Patent 4,946,778) can be adapted to produce single chain antibodies to proteins of the present invention.

25 For polyclonal antibodies, antibody containing antisera is isolated from the immunized animal and is screened for the presence of antibodies with the desired specificity using one of the above-described procedures.

30 The present invention further provides the above-described antibodies in detectably labelled form. Antibodies can be detectably labelled through the use of radioisotopes, affinity labels (such as biotin, avidin, *etc.*), enzymatic labels (such as horseradish peroxidase, alkaline phosphatase, *etc.*) fluorescent labels (such as FITC or rhodamine, *etc.*), paramagnetic atoms, *etc.* Procedures for accomplishing such labeling are well-known in the art, for example see Sternberger *et al.*, *J. Histochem. Cytochem.* 18:315 (1970); Bayer, E. A. *et al.*, *Meth. Enzym.* 62:308

(1979); Engval, E. *et al.*, *Immunol.* 109:129 (1972); Goding, J. W., *J. Immunol. Meth.* 13:215 (1976)).

The labeled antibodies of the present invention can be used for *in vitro*, *in vivo*, and in situ assays to identify cells or tissues in which a fragment of the
5 *Streptococcus pneumoniae* genome is expressed.

The present invention further provides the above-described antibodies immobilized on a solid support. Examples of such solid supports include plastics such as polycarbonate, complex carbohydrates such as agarose and sepharose, acrylic resins and such as polyacrylamide and latex beads. Techniques for
10 coupling antibodies to such solid supports are well known in the art (Weir, D. M. *et al.*, "Handbook of Experimental Immunology" 4th Ed., Blackwell Scientific Publications, Oxford, England, Chapter 10 (1986); Jacoby, W. D. *et al.*, *Meth. Enzym.* 34 Academic Press, N. Y. (1974)). The immobilized antibodies of the present invention can be used for *in vitro*, *in vivo*, and in situ assays as well as for
15 immunoaffinity purification of the proteins of the present invention.

3. Diagnostic Assays and Kits

The present invention further provides methods to identify the expression of one of the ORFs of the present invention, or homolog thereof, in a test sample,
20 using one of the DFs or antibodies of the present invention.

In detail, such methods comprise incubating a test sample with one or more of the antibodies or one or more of the DFs of the present invention and assaying for binding of the DFs or antibodies to components within the test sample.

Conditions for incubating a DF or antibody with a test sample vary.
25 Incubation conditions depend on the format employed in the assay, the detection methods employed, and the type and nature of the DF or antibody used in the assay. One skilled in the art will recognize that any one of the commonly available hybridization, amplification or immunological assay formats can readily be adapted to employ the DFs or antibodies of the present invention. Examples of such assays
30 can be found in Chard, T., *An Introduction to Radioimmunoassay and Related Techniques*, Elsevier Science Publishers, Amsterdam, The Netherlands (1986); Bullock, G. R. *et al.*, *Techniques in Immunocytochemistry*, Academic Press, Orlando, FL Vol. 1 (1982), Vol. 2 (1983), Vol. 3 (1985); Tijssen, P., *Practice and Theory of Enzyme Immunoassays: Laboratory Techniques in Biochemistry and*

Molecular Biology, Elsevier Science Publishers, Amsterdam, The Netherlands (1985).

The test samples of the present invention include cells, protein or membrane extracts of cells, or biological fluids such as sputum, blood, serum, plasma, or urine. The test sample used in the above-described method will vary based on the assay format, nature of the detection method and the tissues, cells or extracts used as the sample to be assayed. Methods for preparing protein extracts or membrane extracts of cells are well known in the art and can be readily be adapted in order to obtain a sample which is compatible with the system utilized.

In another embodiment of the present invention, kits are provided which contain the necessary reagents to carry out the assays of the present invention.

Specifically, the invention provides a compartmentalized kit to receive, in close confinement, one or more containers which comprises: (a) a first container comprising one of the DFs or antibodies of the present invention; and (b) one or more other containers comprising one or more of the following: wash reagents, reagents capable of detecting presence of a bound DF or antibody.

In detail, a compartmentalized kit includes any kit in which reagents are contained in separate containers. Such containers include small glass containers, plastic containers or strips of plastic or paper. Such containers allows one to efficiently transfer reagents from one compartment to another compartment such that the samples and reagents are not cross-contaminated, and the agents or solutions of each container can be added in a quantitative fashion from one compartment to another. Such containers will include a container which will accept the test sample, a container which contains the antibodies used in the assay, containers which contain wash reagents (such as phosphate buffered saline, Tris-buffers, *etc.*), and containers which contain the reagents used to detect the bound antibody or DF.

Types of detection reagents include labelled nucleic acid probes, labelled secondary antibodies, or in the alternative, if the primary antibody is labelled, the enzymatic, or antibody binding reagents which are capable of reacting with the labelled antibody. One skilled in the art will readily recognize that the disclosed DFs and antibodies of the present invention can be readily incorporated into one of the established kit formats which are well known in the art.

4. Screening Assay for Binding Agents

Using the isolated proteins of the present invention, the present invention further provides methods of obtaining and identifying agents which bind to a protein encoded by one of the ORFs of the present invention or to one of the fragments and the *Streptococcus pneumoniae* fragment and contigs herein
5 described.

In general, such methods comprise steps of:

(a) contacting an agent with an isolated protein encoded by one of the ORFs of the present invention, or an isolated fragment of the *Streptococcus pneumoniae* genome; and

10 (b) determining whether the agent binds to said protein or said fragment.

The agents screened in the above assay can be, but are not limited to, peptides, carbohydrates, vitamin derivatives, or other pharmaceutical agents. The agents can be selected and screened at random or rationally selected or designed using protein modeling techniques.

15 For random screening, agents such as peptides, carbohydrates, pharmaceutical agents and the like are selected at random and are assayed for their ability to bind to the protein encoded by the ORF of the present invention.

Alternatively, agents may be rationally selected or designed. As used herein, an agent is said to be "rationally selected or designed" when the agent is
20 chosen based on the configuration of the particular protein. For example, one skilled in the art can readily adapt currently available procedures to generate peptides, pharmaceutical agents and the like capable of binding to a specific peptide sequence in order to generate rationally designed antipeptide peptides, for example see Hurby *et al.*, "Application of Synthetic Peptides: Antisense Peptides," in
25 *Synthetic Peptides, A User's Guide*, W. H. Freeman, NY (1992), pp. 289-307, and Kaspczak *et al.*, *Biochemistry* 28:9230-8 (1989), or pharmaceutical agents, or the like.

In addition to the foregoing, one class of agents of the present invention, as broadly described, can be used to control gene expression through binding to one
30 of the ORFs or EMFs of the present invention. As described above, such agents can be randomly screened or rationally designed/selected. Targeting the ORF or EMF allows a skilled artisan to design sequence specific or element specific agents, modulating the expression of either a single ORF or multiple ORFs which rely on the same EMF for expression control.

One class of DNA binding agents are agents which contain base residues which hybridize or form a triple helix by binding to DNA or RNA. Such agents can be based on the classic phosphodiester, ribonucleic acid backbone, or can be a variety of sulfhydryl or polymeric derivatives which have base attachment capacity.

5 Agents suitable for use in these methods usually contain 20 to 40 bases and are designed to be complementary to a region of the gene involved in transcription (triple helix - see Lee *et al.*, *Nucl. Acids Res.* 6:3073 (1979); Cooney *et al.*, *Science* 241:456 (1988); and Dervan *et al.*, *Science* 251:1360 (1991)) or to the mRNA itself (antisense - Okano, *J. Neurochem.* 56:560 (1991);
10 *Oligodeoxynucleotides as Antisense Inhibitors of Gene Expression*, CRC Press, Boca Raton, FL (1988)). Triple helix- formation optimally results in a shut-off of RNA transcription from DNA, while antisense RNA hybridization blocks translation of an mRNA molecule into polypeptide. Both techniques have been demonstrated to be effective in model systems. Information contained in the
15 sequences of the present invention can be used to design antisense and triple helix-forming oligonucleotides, and other DNA binding agents.

5. Pharmaceutical Compositions and Vaccines

The present invention further provides pharmaceutical agents which can be
20 used to modulate the growth or pathogenicity of *Streptococcus pneumoniae*, or another related organism, *in vivo* or *in vitro*. As used herein, a "pharmaceutical agent" is defined as a composition of matter which can be formulated using known techniques to provide a pharmaceutical compositions. As used herein, the "pharmaceutical agents of the present invention" refers the pharmaceutical agents
25 which are derived from the proteins encoded by the ORFs of the present invention or are agents which are identified using the herein described assays.

As used herein, a pharmaceutical agent is said to "modulate the growth pathogenicity of *Streptococcus pneumoniae* or a related organism, *in vivo* or *in vitro*," when the agent reduces the rate of growth, rate of division, or viability of
30 the organism in question. The pharmaceutical agents of the present invention can modulate the growth or pathogenicity of an organism in many fashions, although an understanding of the underlying mechanism of action is not needed to practice the use of the pharmaceutical agents of the present invention. Some agents will modulate the growth by binding to an important protein thus blocking the biological
35 activity of the protein, while other agents may bind to a component of the outer

surface of the organism blocking attachment or rendering the organism more prone to act the bodies nature immune system. Alternatively, the agent may comprise a protein encoded by one of the ORFs of the present invention and serve as a vaccine. The development and use of a vaccine based on outer membrane components are well known in the art.

As used herein, a "related organism" is a broad term which refers to any organism whose growth can be modulated by one of the pharmaceutical agents of the present invention. In general, such an organism will contain a homolog of the protein which is the target of the pharmaceutical agent or the protein used as a vaccine. As such, related organisms do not need to be bacterial but may be fungal or viral pathogens.

The pharmaceutical agents and compositions of the present invention may be administered in a convenient manner, such as by the oral, topical, intravenous, intraperitoneal, intramuscular, subcutaneous, intranasal or intradermal routes. The pharmaceutical compositions are administered in an amount which is effective for treating and/or prophylaxis of the specific indication. In general, they are administered in an amount of at least about 1 mg/kg body weight and in most cases they will be administered in an amount not in excess of about 1 g/kg body weight per day. In most cases, the dosage is from about 0.1 mg/kg to about 10 g/kg body weight daily, taking into account the routes of administration, symptoms, *etc.*

The agents of the present invention can be used in native form or can be modified to form a chemical derivative. As used herein, a molecule is said to be a "chemical derivative" of another molecule when it contains additional chemical moieties not normally a part of the molecule. Such moieties may improve the molecule's solubility, absorption, biological half life, *etc.* The moieties may alternatively decrease the toxicity of the molecule, eliminate or attenuate any undesirable side effect of the molecule, *etc.* Moieties capable of mediating such effects are disclosed in, among other sources, REMINGTON'S PHARMACEUTICAL SCIENCES (1980) cited elsewhere herein.

For example, such moieties may change an immunological character of the functional derivative, such as affinity for a given antibody. Such changes in immunomodulation activity are measured by the appropriate assay, such as a competitive type immunoassay. Modifications of such protein properties as redox or thermal stability, biological half-life, hydrophobicity, susceptibility to proteolytic degradation or the tendency to aggregate with carriers or into multimers also may

be effected in this way and can be assayed by methods well known to the skilled artisan.

The therapeutic effects of the agents of the present invention may be obtained by providing the agent to a patient by any suitable means (*e.g.*, inhalation, intravenously, intramuscularly, subcutaneously, enterally, or parenterally). It is preferred to administer the agent of the present invention so as to achieve an effective concentration within the blood or tissue in which the growth of the organism is to be controlled. To achieve an effective blood concentration, the preferred method is to administer the agent by injection. The administration may be by continuous infusion, or by single or multiple injections.

In providing a patient with one of the agents of the present invention, the dosage of the administered agent will vary depending upon such factors as the patient's age, weight, height, sex, general medical condition, previous medical history, *etc.* In general, it is desirable to provide the recipient with a dosage of agent which is in the range of from about 1 pg/kg to 10 mg/kg (body weight of patient), although a lower or higher dosage may be administered. The therapeutically effective dose can be lowered by using combinations of the agents of the present invention or another agent.

As used herein, two or more compounds or agents are said to be administered "in combination" with each other when either (1) the physiological effects of each compound, or (2) the serum concentrations of each compound can be measured at the same time. The composition of the present invention can be administered concurrently with, prior to, or following the administration of the other agent.

The agents of the present invention are intended to be provided to recipient subjects in an amount sufficient to decrease the rate of growth (as defined above) of the target organism.

The administration of the agent(s) of the invention may be for either a "prophylactic" or "therapeutic" purpose. When provided prophylactically, the agent(s) are provided in advance of any symptoms indicative of the organisms growth. The prophylactic administration of the agent(s) serves to prevent, attenuate, or decrease the rate of onset of any subsequent infection. When provided therapeutically, the agent(s) are provided at (or shortly after) the onset of an indication of infection. The therapeutic administration of the compound(s)

serves to attenuate the pathological symptoms of the infection and to increase the rate of recovery.

The agents of the present invention are administered to a subject, such as a mammal, or a patient, in a pharmaceutically acceptable form and in a therapeutically effective concentration. A composition is said to be "pharmacologically acceptable" if its administration can be tolerated by a recipient patient. Such an agent is said to be administered in a "therapeutically effective amount" if the amount administered is physiologically significant. An agent is physiologically significant if its presence results in a detectable change in the physiology of a recipient patient.

The agents of the present invention can be formulated according to known methods to prepare pharmaceutically useful compositions, whereby these materials, or their functional derivatives, are combined in a mixture with a pharmaceutically acceptable carrier vehicle. Suitable vehicles and their formulation, inclusive of other human proteins, *e.g.*, human serum albumin, are described, for example, in REMINGTON'S PHARMACEUTICAL SCIENCES, 16th Ed., Osol, A., Ed., Mack Publishing, Easton PA (1980). In order to form a pharmaceutically acceptable composition suitable for effective administration, such compositions will contain an effective amount of one or more of the agents of the present invention, together with a suitable amount of carrier vehicle.

Additional pharmaceutical methods may be employed to control the duration of action. Control release preparations may be achieved through the use of polymers to complex or absorb one or more of the agents of the present invention. The controlled delivery may be effectuated by a variety of well known techniques, including formulation with macromolecules such as, for example, polyesters, polyamino acids, polyvinyl, pyrrolidone, ethylenevinylacetate, methylcellulose, carboxymethylcellulose, or protamine, sulfate, adjusting the concentration of the macromolecules and the agent in the formulation, and by appropriate use of methods of incorporation, which can be manipulated to effectuate a desired time course of release. Another possible method to control the duration of action by controlled release preparations is to incorporate agents of the present invention into particles of a polymeric material such as polyesters, polyamino acids, hydrogels, poly(lactic acid) or ethylene vinylacetate copolymers. Alternatively, instead of incorporating these agents into polymeric particles, it is possible to entrap these materials in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization with, for example, hydroxymethylcellulose or gelatine-

microcapsules and poly(methylmethacrylate) microcapsules, respectively, or in colloidal drug delivery systems, for example, liposomes, albumin microspheres, microemulsions, nanoparticles, and nanocapsules or in macroemulsions. Such techniques are disclosed in REMINGTON'S PHARMACEUTICAL SCIENCES
5 (1980).

The invention further provides a pharmaceutical pack or kit comprising one or more containers filled with one or more of the ingredients of the pharmaceutical compositions of the invention. Associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or
10 sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration.

In addition, the agents of the present invention may be employed in conjunction with other therapeutic compounds.

15 6. Shot-Gun Approach to Megabase DNA Sequencing

The present invention further demonstrates that a large sequence can be sequenced using a random shotgun approach. This procedure, described in detail in the examples that follow, has eliminated the up front cost of isolating and ordering overlapping or contiguous subclones prior to the start of the sequencing
20 protocols.

Certain aspects of the present invention are described in greater detail in the examples that follow. The examples are provided by way of illustration. Other aspects and embodiments of the present invention are contemplated by the inventors, as will be clear to those of skill in the art from reading the present
25 disclosure.

ILLUSTRATIVE EXAMPLES

LIBRARIES AND SEQUENCING

30 1. Shotgun Sequencing Probability Analysis

The overall strategy for a shotgun approach to whole genome sequencing follows from the Lander and Waterman (Landerman and Waterman, *Genomics* 2:231 (1988)) application of the equation for the Poisson distribution. According to this treatment, the probability, P , that any given base in a sequence of size L , in
35 nucleotides, is not sequenced after a certain amount, n , in nucleotides, of random

sequence has been determined can be calculated by the equation $P = e^{-m}$, where m is L/n , the fold coverage. For instance, for a genome of 2.8 Mb, $m=1$ when 2.8 Mb of sequence has been randomly generated (1X coverage). At that point, $P = e^{-1} = 0.37$. The probability that any given base has not been sequenced is the same as the probability that any region of the whole sequence L has not been determined and, therefore, is equivalent to the fraction of the whole sequence that has yet to be determined. Thus, at one-fold coverage, approximately 37% of a polynucleotide of size L , in nucleotides has not been sequenced. When 14 Mb of sequence has been generated, coverage is 5X for a 2.8 Mb and the unsequenced fraction drops to .0067 or 0.67%. 5X coverage of a 2.8 Mb sequence can be attained by sequencing approximately 17,000 random clones from both insert ends with an average sequence read length of 410 bp.

Similarly, the total gap length, G , is determined by the equation $G = Le^{-m}$, and the average gap size, g , follows the equation, $g = L/n$. Thus, 5X coverage leaves about 240 gaps averaging about 82 bp in size in a sequence of a polynucleotide 2.8 Mb long.

The treatment above is essentially that of Lander and Waterman, *Genomics* 2: 231 (1988).

2. Random Library Construction

In order to approximate the random model described above during actual sequencing, a nearly ideal library of cloned genomic fragments is required. The following library construction procedure was developed to achieve this end.

Streptococcus pneumoniae DNA is prepared by phenol extraction. A mixture containing 200 µg DNA in 1.0 ml of 300 mM sodium acetate, 10 mM Tris-HCl, 1 mM Na-EDTA, 50% glycerol is processed through a nebulizer (IPI Medical Products) with a stream of nitrogen adjusted to 35 Kpa for 2 minutes. The sonicated DNA is ethanol precipitated and redissolved in 500 µl TE buffer.

To create blunt-ends, a 100 µl aliquot of the resuspended DNA is digested with 5 units of BAL31 nuclease (New England BioLabs) for 10 min at 30°C in 200 µl BAL31 buffer. The digested DNA is phenol-extracted, ethanol-precipitated, redissolved in 100 µl TE buffer, and then size-fractionated by electrophoresis through a 1.0% low melting temperature agarose gel. The section containing DNA fragments 1.6-2.0 kb in size is excised from the gel, and the LGT agarose is melted and the resulting solution is extracted with phenol to separate the agarose from the

DNA. DNA is ethanol precipitated and redissolved in 20 µl of TE buffer for ligation to vector.

A two-step ligation procedure is used to produce a plasmid library with 97% inserts, of which >99% were single inserts. The first ligation mixture (50 µl)
5 contains 2 µg of DNA fragments, 2 µg pUC18 DNA (Pharmacia) cut with SmaI and dephosphorylated with bacterial alkaline phosphatase, and 10 units of T4 ligase (GIBCO/BRL) and is incubated at 14°C for 4 hr. The ligation mixture then is phenol extracted and ethanol precipitated, and the precipitated DNA is dissolved in 20 µl TE buffer and electrophoresed on a 1.0% low melting agarose gel. Discrete
10 bands in a ladder are visualized by ethidium bromide-staining and UV illumination and identified by size as insert (I), vector (v), v+I, v+2i, v+3i, etc. The portion of the gel containing v+I DNA is excised and the v+I DNA is recovered and resuspended into 20 µl TE. The v+I DNA then is blunt-ended by T4 polymerase treatment for 5 min. at 37°C in a reaction mixture (50 µl) containing the v+I linears,
15 500 µM each of the 4 dNTPs, and 9 units of T4 polymerase (New England BioLabs), under recommended buffer conditions. After phenol extraction and ethanol precipitation the repaired v+I linears are dissolved in 20 µl TE. The final ligation to produce circles is carried out in a 50 µl reaction containing 5 µl of v+I linears and 5 units of T4 ligase at 14°C overnight. After 10 min. at 70°C the
20 following day, the reaction mixture is stored at -20°C.

This two-stage procedure results in a molecularly random collection of single-insert plasmid recombinants with minimal contamination from double-insert chimeras (<1%) or free vector (<3%).

Since deviation from randomness can arise from propagation the DNA in the host, *E. coli* host cells deficient in all recombination and restriction functions
25 (A. Greener, *Strategies* 3 (1):5 (1990)) are used to prevent rearrangements, deletions, and loss of clones by restriction. Furthermore, transformed cells are plated directly on antibiotic diffusion plates to avoid the usual broth recovery phase which allows multiplication and selection of the most rapidly growing cells.

30 Plating is carried out as follows. A 100 µl aliquot of Epicurian Coli SURE II Supercompetent Cells (Stratagene 200152) is thawed on ice and transferred to a chilled Falcon 2059 tube on ice. A 1.7 µl aliquot of 1.42 M beta-mercaptoethanol is added to the aliquot of cells to a final concentration of 25 mM. Cells are incubated on ice for 10 min. A 1 µl aliquot of the final ligation is added to the cells
35 and incubated on ice for 30 min. The cells are heat pulsed for 30 sec. at 42°C and

placed back on ice for 2 min. The outgrowth period in liquid culture is eliminated from this protocol in order to minimize the preferential growth of any given transformed cell. Instead the transformation mixture is plated directly on a nutrient rich SOB plate containing a 5 ml bottom layer of SOB agar (5% SOB agar: 20 g
5 tryptone, 5 g yeast extract, 0.5 g NaCl, 1.5% Difco Agar per liter of media). The 5 ml bottom layer is supplemented with 0.4 ml of 50 mg/ml ampicillin per 100 ml SOB agar. The 15 ml top layer of SOB agar is supplemented with 1 ml X-Gal (2%), 1 ml MgCl₂ (1 M), and 1 ml MgSO₄ /100 ml SOB agar. The 15 ml top layer is poured just prior to plating. Our titer is approximately 100 colonies/10 μ l aliquot
10 of transformation.²⁴

All colonies are picked for template preparation regardless of size. Thus, only clones lost due to "poison" DNA or deleterious gene products are deleted from the library, resulting in a slight increase in gap number over that expected.

15 3. Random DNA Sequencing

High quality double stranded DNA plasmid templates are prepared using a "boiling bead" method developed in collaboration with Advanced Genetic Technology Corp. (Gaithersburg, MD) (Adams *et al.*, *Science* 252:1651 (1991); Adams *et al.*, *Nature* 355:632 (1992)). Plasmid preparation is performed in a 96-
20 well format for all stages of DNA preparation from bacterial growth through final DNA purification. Template concentration is determined using Hoechst Dye and a Millipore Cytofluor. DNA concentrations are not adjusted, but low-yielding templates are identified where possible and not sequenced.

Templates are also prepared from two *Streptococcus pneumoniae* lambda
25 genomic libraries. An amplified library is constructed in the vector Lambda GEM-12 (Promega) and an unamplified library is constructed in Lambda DASH II (Stratagene). In particular, for the unamplified lambda library, *Streptococcus pneumoniae* DNA (> 100 kb) is partially digested in a reaction mixture (200 μ l) containing 50 μ g DNA, 1X Sau3AI buffer, 20 units Sau3AI for 6 min. at 23°C.
30 The digested DNA was phenol-extracted and electrophoresed on a 0.5% low melting agarose gel at 2V/cm for 7 hours. Fragments from 15 to 25 kb are excised and recovered in a final volume of 6 μ l. One μ l of fragments is used with 1 μ l of DASHII vector (Stratagene) in the recommended ligation reaction. One μ l of the ligation mixture is used per packaging reaction following the recommended
35 protocol with the Gigapack II XL Packaging Extract (Stratagene, #227711). Phage

are plated directly without amplification from the packaging mixture (after dilution with 500 μ l of recommended SM buffer and chloroform treatment). Yield is about 2.5×10^3 pfu/ul. The amplified library is prepared essentially as above except the lambda GEM-12 vector is used. After packaging, about 3.5×10^4 pfu are plated on the restrictive NM539 host. The lysate is harvested in 2 ml of SM buffer and stored frozen in 7% dimethylsulfoxide. The phage titer is approximately 1×10^9 pfu/ml.

Liquid lysates (100 μ l) are prepared from randomly selected plaques (from the unamplified library) and template is prepared by long-range PCR using T7 and T3 vector-specific primers.

Sequencing reactions are carried out on plasmid and/or PCR templates using the AB Catalyst LabStation with Applied Biosystems PRISM Ready Reaction Dye Primer Cycle Sequencing Kits for the M13 forward (M13-21) and the M13 reverse (M13RP1) primers (Adams *et al.*, *Nature* 368:474 (1994)). Dye terminator sequencing reactions are carried out on the lambda templates on a Perkin-Elmer 9600 Thermocycler using the Applied Biosystems Ready Reaction Dye Terminator Cycle Sequencing kits. T7 and SP6 primers are used to sequence the ends of the inserts from the Lambda GEM-12 library and T7 and T3 primers are used to sequence the ends of the inserts from the Lambda DASH II library. Sequencing reactions are performed by eight individuals using an average of fourteen AB 373 DNA Sequencers per day. All sequencing reactions are analyzed using the Stretch modification of the AB 373, primarily using a 34 cm well-to-read distance. The overall sequencing success rate very approximately is about 85% for M13-21 and M13RP1 sequences and 65% for dye-terminator reactions. The average usable read length is 485 bp for M13-21 sequences, 445bp for M13RP1 sequences, and 375 bp for dye-terminator reactions.

Richards *et al.*, Chapter 28 in AUTOMATED DNA SEQUENCING AND ANALYSIS, M. D. Adams, C. Fields, J. C. Venter, Eds., Academic Press, London, (1994) described the value of using sequence from both ends of sequencing templates to facilitate ordering of contigs in shotgun assembly projects of lambda and cosmid clones. We balance the desirability of both-end sequencing (including the reduced cost of lower total number of templates) against shorter read-lengths for sequencing reactions performed with the M13RP1 (reverse) primer compared to the M13-21 (forward) primer. Approximately one-half of the templates are sequenced from both ends. Random reverse sequencing reactions are

done based on successful forward sequencing reactions. Some M13RP1 sequences are obtained in a semi-directed fashion: M13-21: sequences pointing outward at the ends of contigs are chosen for M13RP1 sequencing in an effort to specifically order contigs.

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4. Protocol for Automated Cycle Sequencing

The sequencing is carried out using ABI Catalyst robots and AB 373 Automated DNA Sequencers. The Catalyst robot is a publicly available sophisticated pipetting and temperature control robot which has been developed specifically for DNA sequencing reactions. The Catalyst combines pre-aliquoted templates and reaction mixes consisting of deoxy- and dideoxynucleotides, the thermostable Taq DNA polymerase, fluorescently-labelled sequencing primers, and reaction buffer. Reaction mixes and templates are combined in the wells of an aluminum 96-well thermocycling plate. Thirty consecutive cycles of linear amplification (*i.e.*, one primer synthesis) steps are performed including denaturation, annealing of primer and template, and extension; *i.e.*, DNA synthesis. A heated lid with rubber gaskets on the thermocycling plate prevents evaporation without the need for an oil overlay.

Two sequencing protocols are used: one for dye-labelled primers and a second for dye-labelled dideoxy chain terminators. The shotgun sequencing involves use of four dye-labelled sequencing primers, one for each of the four terminator nucleotide. Each dye-primer is labelled with a different fluorescent dye, permitting the four individual reactions to be combined into one lane of the 373 DNA Sequencer for electrophoresis, detection, and base-calling. ABI currently supplies pre-mixed reaction mixes in bulk packages containing all the necessary non-template reagents for sequencing. Sequencing can be done with both plasmid and PCR- generated templates with both dye-primers and dye- terminators with approximately equal fidelity, although plasmid templates generally give longer usable sequences.

Thirty-two reactions are loaded per AB373 Sequencer each day, for a total of 960 samples. Electrophoresis is run overnight following the manufacturer's protocols, and the data is collected for twelve hours. Following electrophoresis and fluorescence detection, the ABI 373 performs automatic lane tracking and base-calling. The lane-tracking is confirmed visually. Each sequence electropherogram (or fluorescence lane trace) is inspected visually and assessed for quality. Trailing

sequences of low quality are removed and the sequence itself is loaded via software to a Sybase database (archived daily to 8mm tape). Leading vector polylinker sequence is removed automatically by a software program. Average edited lengths of sequences from the standard ABI 373 are around 400 bp and depend mostly on the quality of the template used for the sequencing reaction. ABI 373 Sequencers converted to Stretch Liners provide a longer electrophoresis path prior to fluorescence detection and increase the average number of usable bases to 500-600 bp.

10 INFORMATICS

1. Data Management

A number of information management systems for a large-scale sequencing lab have been developed. (For review see, for instance, Kerlavage *et al.*, *Proceedings of the Twenty-Sixth Annual Hawaii International Conference on System Sciences*, IEEE Computer Society Press, Washington D. C., 585 (1993)) The system used to collect and assemble the sequence data was developed using the Sybase relational database management system and was designed to automate data flow wherever possible and to reduce user error. The database stores and correlates all information collected during the entire operation from template preparation to final analysis of the genome. Because the raw output of the ABI 373 Sequencers was based on a Macintosh platform and the data management system chosen was based on a Unix platform, it was necessary to design and implement a variety of multi- user, client-server applications which allow the raw data as well as analysis results to flow seamlessly into the database with a minimum of user effort.

25

2. Assembly

An assembly engine (TIGR Assembler) developed for the rapid and accurate assembly of thousands of sequence fragments was employed to generate contigs. The TIGR assembler simultaneously clusters and assembles fragments of the genome. In order to obtain the speed necessary to assemble more than 10^4 fragments, the algorithm builds a hash table of 12 bp oligonucleotide subsequences to generate a list of potential sequence fragment overlaps. The number of potential overlaps for each fragment determines which fragments are likely to fall into repetitive elements. Beginning with a single seed sequence fragment, TIGR Assembler extends the current contig by attempting to add the best matching

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fragment based on oligonucleotide content. The contig and candidate fragment are aligned using a modified version of the Smith-Waterman algorithm which provides for optimal gapped alignments (Waterman, M. S., *Methods in Enzymology* 164:765 (1988)). The contig is extended by the fragment only if strict criteria for the quality of the match are met. The match criteria include the minimum length of overlap, the maximum length of an unmatched end, and the minimum percentage match. These criteria are automatically lowered by the algorithm in regions of minimal coverage and raised in regions with a possible repetitive element. The number of potential overlaps for each fragment determines which fragments are likely to fall into repetitive elements. Fragments representing the boundaries of repetitive elements and potentially chimeric fragments are often rejected based on partial mismatches at the ends of alignments and excluded from the current contig. TIGR Assembler is designed to take advantage of clone size information coupled with sequencing from both ends of each template. It enforces the constraint that sequence fragments from two ends of the same template point toward one another in the contig and are located within a certain range of base pairs (definable for each clone based on the known clone size range for a given library).

The process resulted in 391 contigs as represented by SEQ ID NOs:1-391.

3. Identifying Genes

The predicted coding regions of the *Streptococcus pneumoniae* genome were initially defined with the program GeneMark, which finds ORFs using a probabilistic classification technique. The predicted coding region sequences were used in searches against a database of all nucleotide sequences from GenBank (October, 1997), using the BLASTN search method to identify overlaps of 50 or more nucleotides with at least a 95% identity. Those ORFs with nucleotide sequence matches are shown in Table 1. The ORFs without such matches were translated to protein sequences and compared to a non-redundant database of known proteins generated by combining the Swiss-prot, PIR and GenPept databases. ORFs that matched a database protein with BLASTP probability less than or equal to 0.01 are shown in Table 2. The table also lists assigned functions based on the closest match in the databases. ORFs that did not match protein or nucleotide sequences in the databases at these levels are shown in Table 3.

ILLUSTRATIVE APPLICATIONS

1. Production of an Antibody to a *Streptococcus pneumoniae* Protein

Substantially pure protein or polypeptide is isolated from the transfected or transformed cells using any one of the methods known in the art. The protein can also be produced in a recombinant prokaryotic expression system, such as *E. coli*, or can be chemically synthesized. Concentration of protein in the final preparation is adjusted, for example, by concentration on an Amicon filter device, to the level of a few micrograms/ml. Monoclonal or polyclonal antibody to the protein can then be prepared as follows.

2. Monoclonal Antibody Production by Hybridoma Fusion

Monoclonal antibody to epitopes of any of the peptides identified and isolated as described can be prepared from murine hybridomas according to the classical method of Kohler, G. and Milstein, C., *Nature* 256:495 (1975) or modifications of the methods thereof. Briefly, a mouse is repetitively inoculated with a few micrograms of the selected protein over a period of a few weeks. The mouse is then sacrificed, and the antibody producing cells of the spleen isolated. The spleen cells are fused by means of polyethylene glycol with mouse myeloma cells, and the excess unfused cells destroyed by growth of the system on selective media comprising aminopterin (HAT media). The successfully fused cells are diluted and aliquots of the dilution placed in wells of a microtiter plate where growth of the culture is continued. Antibody-producing clones are identified by detection of antibody in the supernatant fluid of the wells by immunoassay procedures, such as ELISA, as originally described by Engvall, E., *Meth. Enzymol.* 70:419 (1980), and modified methods thereof. Selected positive clones can be expanded and their monoclonal antibody product harvested for use. Detailed procedures for monoclonal antibody production are described in Davis, L. *et al.*, *Basic Methods in Molecular Biology*, Elsevier, New York. Section 21-2 (1989).

3. Polyclonal Antibody Production by Immunization

5 Polyclonal antiserum containing antibodies to heterogenous epitopes of a single protein can be prepared by immunizing suitable animals with the expressed protein described above, which can be unmodified or modified to enhance immunogenicity. Effective polyclonal antibody production is affected by many factors related both to the antigen and the host species. For example, small molecules tend to be less immunogenic than others and may require the use of carriers and adjuvant. Also, host animals vary in response to site of inoculations and dose, with both inadequate or excessive doses of antigen resulting in low titer antisera. Small doses (ng level) of antigen administered at multiple intradermal sites appears to be most reliable. An effective immunization protocol for rabbits can be found in Vaitukaitis, J. *et al.*, *J. Clin. Endocrinol. Metab.* 33:988-991 (1971).

15 Booster injections can be given at regular intervals, and antiserum harvested when antibody titer thereof, as determined semi-quantitatively, for example, by double immunodiffusion in agar against known concentrations of the antigen, begins to fall. See, for example, Ouchterlony, O. *et al.*, Chap. 19 in: *Handbook of Experimental Immunology*, Wier, D., ed, Blackwell (1973). Plateau concentration of antibody is usually in the range of 0.1 to 0.2 mg/ml of serum (about 12M). Affinity of the antisera for the antigen is determined by preparing competitive binding curves, as described, for example, by Fisher, D., Chap. 42 in: *Manual of Clinical Immunology*, second edition, Rose and Friedman, eds., Amer. Soc. For Microbiology, Washington, D. C. (1980)

25 Antibody preparations prepared according to either protocol are useful in quantitative immunoassays which determine concentrations of antigen-bearing substances in biological samples; they are also used semi- quantitatively or qualitatively to identify the presence of antigen in a biological sample. In addition, antibodies are useful in various animal models of pneumococcal disease as a means of evaluating the protein used to make the antibody as a potential vaccine target or as a means of evaluating the antibody as a potential immunotherapeutic or immunoprophylactic reagent.

4. Preparation of PCR Primers and Amplification of DNA

Various fragments of the *Streptococcus pneumoniae* genome, such as those of Tables 1-3 and SEQ ID NOS:1-391 can be used, in accordance with the present invention, to prepare PCR primers for a variety of uses. The PCR primers are preferably at least 15 bases, and more preferably at least 18 bases in length. When selecting a primer sequence, it is preferred that the primer pairs have approximately the same G/C ratio, so that melting temperatures are approximately the same. The PCR primers and amplified DNA of this Example find use in the Examples that follow.

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5. Gene expression from DNA Sequences Corresponding to ORFs

A fragment of the *Streptococcus pneumoniae* genome provided in Tables 1-3 is introduced into an expression vector using conventional technology. Techniques to transfer cloned sequences into expression vectors that direct protein translation in mammalian, yeast, insect or bacterial expression systems are well known in the art. Commercially available vectors and expression systems are available from a variety of suppliers including Stratagene (La Jolla, California), Promega (Madison, Wisconsin), and Invitrogen (San Diego, California). If desired, to enhance expression and facilitate proper protein folding, the codon context and codon pairing of the sequence may be optimized for the particular expression organism, as explained by Hatfield *et al.*, U. S. Patent No. 5,082,767, incorporated herein by this reference.

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The following is provided as one exemplary method to generate polypeptide(s) from cloned ORFs of the *Streptococcus pneumoniae* genome fragment. Bacterial ORFs generally lack a poly A addition signal. The addition signal sequence can be added to the construct by, for example, splicing out the poly A addition sequence from pSG5 (Stratagene) using BglII and SalI restriction endonuclease enzymes and incorporating it into the mammalian expression vector pXT1 (Stratagene) for use in eukaryotic expression systems. pXT1 contains the LTRs and a portion of the gag gene of Moloney Murine Leukemia Virus. The positions of the LTRs in the construct allow efficient stable transfection. The vector includes the Herpes Simplex thymidine kinase promoter and the selectable neomycin gene. The *Streptococcus pneumoniae* DNA is obtained by PCR from the bacterial vector using oligonucleotide primers complementary to the *Streptococcus pneumoniae* DNA and containing restriction endonuclease sequences for PstI incorporated into the 5' primer and BglII at the 5' end of the corresponding *Streptococcus pneumoniae* DNA 3' primer, taking care to ensure that the *Streptococcus pneumoniae* DNA is positioned such that its followed with the poly A addition sequence. The purified fragment obtained from the resulting PCR reaction is digested with PstI, blunt ended with an exonuclease, digested with BglII, purified and ligated to pXT1, now containing a poly A addition sequence and digested BglII.

The ligated product is transfected into mouse NIH 3T3 cells using Lipofectin (Life Technologies, Inc., Grand Island, New York) under conditions outlined in the product specification. Positive transfectants are selected after growing the transfected cells in 600 ug/ml G418 (Sigma, St. Louis, Missouri). The protein is preferably released into the supernatant. However if the protein has membrane binding domains, the protein may additionally be retained within the cell or expression may be restricted to the cell surface. Since it may be necessary to purify and locate the transfected product, synthetic 15-mer peptides synthesized from the predicted *Streptococcus pneumoniae* DNA sequence are injected into mice to generate antibody to the polypeptide encoded by the *Streptococcus pneumoniae* DNA.

Alternatively and if antibody production is not possible, the *Streptococcus pneumoniae* DNA sequence is additionally incorporated into eukaryotic expression vectors and expressed as, for example, a globin fusion. Antibody to the globin moiety then is used to purify the chimeric protein. Corresponding protease
5 cleavage sites are engineered between the globin moiety and the polypeptide encoded by the *Streptococcus pneumoniae* DNA so that the latter may be freed from the formed by simple protease digestion. One useful expression vector for generating globin chimerics is pSG5 (Stratagene). This vector encodes a rabbit globin. Intron II of the rabbit globin gene facilitates splicing of the expressed
10 transcript, and the polyadenylation signal incorporated into the construct increases the level of expression. These techniques are well known to those skilled in the art of molecular biology. Standard methods are published in methods texts such as Davis *et al.*, cited elsewhere herein, and many of the methods are available from the technical assistance representatives from Stratagene, Life Technologies, Inc., or
15 Promega. Polypeptides of the invention also may be produced using *in vitro* translation systems such as *in vitro* Express™ Translation Kit (Stratagene).

While the present invention has been described in some detail for purposes of clarity and understanding, one skilled in the art will appreciate that various changes in form and detail can be made without departing from the true scope of
20 the invention.

All patents, patent applications and publications referred to above are hereby incorporated by reference.

What Is Claimed Is:

25 1. Computer readable medium having recorded thereon the nucleotide sequence depicted in SEQ ID NOS:1-391, a representative fragment thereof or a nucleotide sequence at least 95% identical to a nucleotide sequence depicted in SEQ ID NOS:1-391.

30 2. Computer readable medium having recorded thereon any one of the fragments of SEQ ID NOS:1-391 depicted in Tables 2 and 3 or a degenerate variant thereof.

35 3. The computer readable medium of claim 1, wherein said medium is selected from the group consisting of a floppy disc, a hard disc, random access memory (RAM), read only memory (ROM), and CD-ROM.

40 4. The computer readable medium of claim 3, wherein said medium is selected from the group consisting of a floppy disc, a hard disc, random access memory (RAM), read only memory (ROM), and CD-ROM.

 5. A computer-based system for identifying fragments of the *Streptococcus pneumoniae* genome of commercial importance comprising the following elements:

45 a) a data storage means comprising the nucleotide sequence of SEQ ID NOS:1-391, a representative fragment thereof, or a nucleotide sequence at least 95% identical to a nucleotide sequence of SEQ ID NOS:1-391;

 b) search means for comparing a target sequence to the nucleotide sequence of the data storage means of step (a) to identify homologous sequence(s), and

50 c) retrieval means for obtaining said homologous sequence(s) of step (b).

55 6. A method for identifying commercially important nucleic acid fragments of the *Streptococcus pneumoniae* genome comprising the step of comparing a database comprising the nucleotide sequences depicted in SEQ ID NOS:1-391, a representative fragment thereof, or a nucleotide sequence at least 95% identical to a nucleotide sequence of SEQ ID NOS:1-391 with a target sequence to obtain a nucleic acid molecule comprised of a complementary nucleotide sequence to said target sequence, wherein said target sequence is not randomly selected.

60 7. A method for identifying an expression modulating fragment of
Streptococcus pneumoniae genome comprising the step of comparing a database
comprising the nucleotide sequences depicted in SEQ ID NOS:1-391, a
representative fragment thereof, or a nucleotide sequence at least 95% identical to
the nucleotide sequence of SEQ ID NOS:1-391 with a target sequence to obtain a
nucleic acid molecule comprised of a complementary nucleotide sequence to said
65 target sequence, wherein said target sequence comprises sequences known to
regulate gene expression.

70 8. An isolated protein-encoding nucleic acid fragment of the *Streptococcus*
pneumoniae genome, wherein said fragment consists of the nucleotide sequence of
any one of the fragments of SEQ ID NOS:1-391 depicted in Tables 2 and 3, or a
degenerate variant thereof.

75 9. A vector comprising any one of the fragments of the *Streptococcus*
pneumoniae genome SEQ ID NOS:1-391 depicted in Tables 2 and 3 or a
degenerate variant thereof.

80 10. An isolated fragment of the *Streptococcus pneumoniae* genome,
wherein said fragment modulates the expression of an operably linked open reading
frame, wherein said fragment consists of the nucleotide sequence from about 10 to
200 bases in length which is 5' to any one of the open reading frames depicted in
Tables 2 and 3 or a degenerate variant thereof.

85 11. A vector comprising any one of the fragments of the *Streptococcus*
pneumoniae genome of claim 8.

12. An organism which has been altered to contain any one of the
fragments of the *Streptococcus pneumoniae* genome of claim 8.

90 13. An organism which has been altered to contain any one of the
fragments of the *Streptococcus pneumoniae* genome of claim 10.

14. A method for regulating the expression of a nucleic acid molecule comprising the step of covalently attaching to said nucleic acid molecule a nucleic acid molecule consisting of the nucleotide sequence from about 10 to 100 bases 5' to any one of the fragments of the *Streptococcus pneumoniae* genome depicted in SEQ ID NOS:1-391 and Tables 2 and 3 or a degenerate variant thereof.

15. An isolated nucleic acid molecule encoding a homolog of any of the fragments of the *Streptococcus pneumoniae* genome of SEQ ID NOS:1-391 and Tables 2 and 3, wherein said nucleic acid molecule is produced by a process comprising steps of:

a) screening a genomic DNA library using as a probe a target sequence defined by any of SEQ ID NOS:1-391 and Tables 2 and 3, including fragments thereof;

b) identifying members of said library which contain sequences that hybridize to said target sequence; and

c) isolating the nucleic acid molecules from said members identified in step (b).

16. An isolated DNA molecule encoding a homolog of any one of the fragments of the *Streptococcus pneumoniae* genome of SEQ ID NOS:1-391 and Tables 2 and 3, wherein said nucleic acid molecule is produced a process comprising steps of:

a) isolating mRNA, DNA, or cDNA produced from an organism;

b) amplifying nucleic acid molecules whose nucleotide sequence is homologous to amplification primers derived from said fragment of said *Streptococcus pneumoniae* genome to prime said amplification;

c) isolating said amplified sequences produced in step (b).

17. An isolated polypeptide encoded by any of the fragments of the *Streptococcus pneumoniae* genome of SEQ ID NOS:1-391 and depicted in Table 2 and 3 or by a degenerate variant of said fragments.

18. An isolated polynucleotide molecule encoding any one of the polypeptides of claim 17.

19. An antibody which selectively binds to any one of the polypeptides of claim 17.

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20. A method for producing a polypeptide in a host cell comprising the steps of:

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a) incubating a host containing a heterologous nucleic acid molecule whose nucleotide sequence consists of any one of the fragments of the *Streptococcus pneumoniae* genome of SEQ ID NOS:1-391 and depicted in Tables 2 and 3, under conditions where said heterologous nucleic acid molecule is expressed to produce said protein, and

b) isolating said protein.

Figure 1

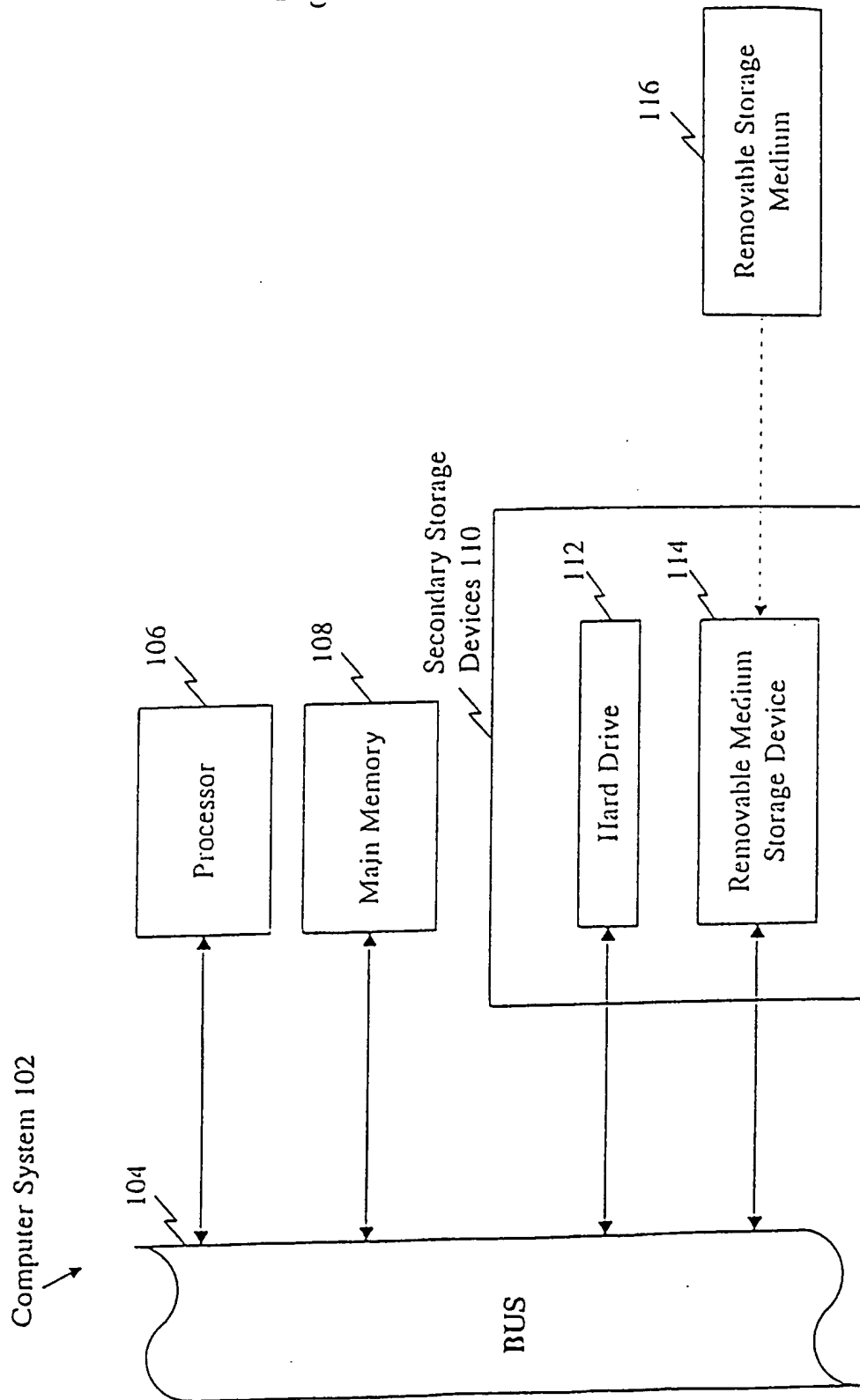


Figure 2

